BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 5/06 — A1

(11) International Publication Number: **WO 00/09667**

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18439

(22) International Filing Date: 13 August 1999 (13.08.99)

(30) Priority Data:
60/096,631 14 August 1998 (14.08.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/096,631 (CON)
Filed on 14 August 1998 (14.08.98)

(71) Applicant *(for all designated States except US)*: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).

(71)(72) Applicants and Inventors: WILLIAMS, R., Sanders [US/US]; 3628 Mockingbird Lane, Dallas, TX 75205 (US). OLSON, Eric, N. [US/US]; 3700 Southwestern Boulevard, Dallas, TX 75225 (US).

(74) Agent: HIGHLANDER, Steven, L.; Arnold White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CALCINEURIN-DEPENDENT CONTROL OF SKELETAL MUSCLE FIBER TYPE

(57) Abstract

The present invention relates to skeletal muscle fiber composition. More particularly, the present invention defines the molecular events linking calcium stimulation to specialization of skeletal muscle fibers. More specifically, the present invention shows that Ca^{2+} stimulation of the slow fiber phenotype is mediated through a calcineurin-dependent pathway. Thus, the present invention provides methods and compositions for altering and/or regulating the phenotype of muscle. Further provided are methods for the detection of compounds having therapeutic activity toward regulating muscle fiber composition.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DESCRIPTION

CALCINEURIN-DEPENDENT CONTROL OF SKELETAL MUSCLE FIBER TYPE

BACKGROUND OF THE INVENTION

This application claims priority to and specifically incoporates by reference, the content of U.S. Provisional Patent Application Serial No. 60/096,631, filed August 14, 1998. The entire text of the above–referenced disclosure is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of molecular biology and physiology. More particularly, it concerns the identification of calcineurin as a central mediator for muscle fiber phenotype.

2. Description of Related Art

There are many disorders in which the muscle fiber type of skeletal muscle plays a role. For example, progressive myonecrosis occurs with advancing age in individuals lacking dystrophin (Duchenne's muscular dystrophy) (Webster *et al.*, 1988). Patients with congestive heart failure, irrespective of the primary cause, exhibit loss of slow, oxidative myofibers in their skeletal muscles (Massie *et al.*, 1988; Sabbah *et al.*, 1993), an abnormality that contributes to exercise intolerance in these individuals. A decline in slow fibers also is observed as a result of prolonged inactivity or hypogravity (Caiozzo *et al.*, 1994), and the fiber composition of skeletal muscles influences insulin sensitivity (Kong *et al.*, 1994) and lipoprotein metabolism (Tikkanen *et al.*, 1996). Thus, the presence of a specific subtype of skeletal myofibers has a defined role in the pathophysiology of various muscular diseases.

Subtypes of skeletal myofibers of adult vertebrates differ markedly with respect to contractile physiology, metabolic capabilities, ultrastructural morphology, and susceptibility to fatigue. The physiological and clinical importance of myofiber specialization has been recognized for several decades, and many studies have identified sets of specific contractile proteins and enzymes of intermediary metabolism, the selective expression of which establishes this physiological and biochemical diversity among skeletal myocytes (Saltin and Gollnick, 1983; Booth and Baldwin, 1996; Schiaffino and Reggiani, 1996).

Fiber type-specific programs of gene expression can be detected at early stages of myogenic development in the embryo (DiMario *et al.*, 1993; Ontell *et al.*, 1993; Stockdale 1997), but remain plastic in adults, where they are subject to modification as a function of contractile load (*e.g.*, exercise training), hormonal shifts, or systemic diseases (Holloszy and Coyle, 1984; Williams and Neufer, 1996; Ianuzzo *et al.*, 1991; Massie *et al.*, 1988; Sabbah *et al.*, 1993).

A central role for motor nerve activity in determining skeletal muscle fiber composition was revealed by cross-innervation and electrical stimulation experiments, which demonstrated complete and reversible transformation of pre-existing myofibers by changing patterns of neuronal firing (Vrbova, 1963; Williams *et al.*, 1986; Pette and Vrbova, 1992). Specifically, brief bursts of neural activity, interspersed between long periods of neuronal quiescence, promote the acquisition of fast-twitch, glycolytic fiber characteristics. Conversely, extended periods of tonic motor nerve activity stimulate a shift to the slow-twitch, oxidative myofiber phenotype.

Neural stimulation provokes changes in the intracellular concentrations of several potential signaling molecules including calcium, cyclic AMP and nitric oxide, as well as immediate early gene products (c-fos) and molecular chaperones (hsp70) (Michel *et al.*, 1994; Neufer *et al.*, 1996; Williams and Neufer, 1996), but specific signaling pathways and regulatory molecules that link motor nerve activity to fiber type-specific gene expression have yet to be identified.

Tonic motor nerve activity at 10-15 Hz is characteristic of slow-twitch fibers (Hennig and Lomo, 1985) and results in a sustained elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$) within a concentration range between 100 and 300 nM (Chin and Allen, 1996), a pattern predicted to activate calcineurin. In fast myofibers, resting $[Ca^{2+}]_i$ is maintained at levels of only 50 nM (Westerblad and Allen, 1991), and the high amplitude (~1M) calcium transients evoked by motor nerve activity are predicted to be of insufficient duration to evoke calcineurin-stimulated signaling. Chronic stimulation at 10 Hz of the motor nerve innervating fast myofibers results in sustained elevations of $[Ca^{2+}]_i$ and promotes fast-to-slow fiber transformation (Williams *et al.*, 1986; Sreter *et al.*, 1987).

Calcineurin-dependent signaling mechanisms have been characterized extensively in the activation of cytokine gene expression in T and B lymphocytes responding to stimuli that elevate $[Ca^{2+}]_i$ (Rao *et al.*, 1997). Calcineurin and several NFAT isoforms are abundant in skeletal muscles (Hoey *et al.*, 1995), though target genes that respond to this pathway in skeletal myocytes have not been identified previously, and a specific role for calcineurin in the control of myofiber specialization has not been previously proposed.

The need to control specific fiber composition of skeletal muscle has significant implications. The elucidation of a signaling pathway linking motor nerve activity to selective changes in gene expression that establish diversity among myofibers will be particularly useful in ameliorating the pathological effects of various muscular diseases, and altering properties of skeletal muscle to prevent or treat systemic diseases such as diabetes and atherosclerosis. The present invention is directed to these and other deficiencies in the art relating to myofiber specialization.

SUMMARY OF THE INVENTION

The present invention provides a method of altering the phenotype of skeletal muscle tissue, comprising contacting the tissue with a modulator of calcineurin activity. In specific embodiments, altering the phenotype comprises an increase of the proportion fast fiber to slow fiber in the tissue. In alternative embodiments, altering the phenotype comprises an increase of the proportion slow fiber to fast fiber in the tissue. In particular aspects of the present invention, the phenotype comprises a change in the size of the cells of the tissue.

In particular embodiments, the muscle cells are derived from soleus, gastroenemius, quadriceps, tibialis anterior, pectordis, lattissimus dorsi, diaphragm, biceps, triceps, gluteus and tongue. Of course, these are exemplary muscle cells and the methods of the present invention may employ any muscle cell in which a fast to slow or slow to fast fiber composition transition is desired, such additional muscle cells are well known to those of skill in the art. In specific embodiments, the muscle cells are soleus muscle cells. The muscle cells may be derived from any animal including but not limited to human, murine, bovine, equine, porcine, ovine, canine, feline, rodent, avian or fish. In more specific embodiments the animal is a human.

In other embodiments, altering the phenotype comprises an increase in expression of fiber-type specific gene expression. More particularly, the fiber-type specific gene expression is specific to fast fiber cells. It is specifically contemplated that the gene expression comprises the expression of muscle creatinine kinase, fast myosin heavy chain, fast myosin light chain, fast troponin or parvalbumin. Alternatively, the fiber-type specific gene expression is specific to slow fiber cells. More particularly, the slow fiber specific gene expression comprises the expression of myoglobin, troponin I, slow myosin heavy chain, slow myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase.

It is particularly contemplated that the modulator is an inhibitor of calcineurin activity. More particularly, the inhibitor of calcineurin reduces the expression of calcineurin. In specific embodiments, the inhibitor of calcineurin is an agent that binds to and inactivates calcineurin. In an alternative embodiments, the inhibitor of calcineurin inhibits the interaction of calcineurin with an NFAT. In yet another embodiment, the agent that reduces the expression of calcineurin is an antisense construct. In those embodiments in which the agent binds to and inactivates calcineurin, such an agent may be an antibody or a small molecule inhibitor. More particularly, the antibody is a single chain antibody. In alternative embodiments, the antibody is a monoclonal antibody. Alternative particular embodiments contemplated that the inhibitor may be selected from the group consisting of cyclosporin, FK506, AP1510 and FK1012. Of course, these are exemplary inhibitors; it is contemplated that additional inhibitors derived from these compounds or acting through the same mechanisms of action as these compounds also will be useful in the context of the present invention. Such derivatives may be naturally occurring variants of these compound, may be produced through rational drug design based upon the structure of these inhibitors, or discovered from small molecule libraries.

In additional embodiments, the modulator may be a stimulator of calcineurin activity. Specifically, it is contemplated that the stimulator of calcineurin activity increases the expression of calcineurin. In other embodiments, the stimulator of calcineurin activity activates the calcineurin. More particularly, the activator of calcineurin activity may be calcium or calmodulin, or any other protein or molecule that acts in a manner to activate calcineurin activity, expression or function. In certain embodiments, the modulator of calcineurin activity increases the activity of an NFAT. In more particular embodiments, the increase in an NFAT

activity comprises stimulation of the dephosphorylation of an NFAT. In certain embodiments, the increase in an NFAT activity comprises increasing the expression of an NFAT. In other embodiments, the increase in an NFAT activity comprises contacting NFAT with an agent that activates the NFAT. In still further embodiments, the increase in an NFAT activity comprises increasing the interaction of NFAT with MEF2. Of course, it is contemplated that the increase in an NFAT activity also may comprises increasing the interaction of NFAT with additional or alternative transcription factors. It is specifically contemplated that the NFAT may be selected from the group consisting of NFAT1, NFAT2, NFAT3 and NFAT4. These are exemplary members of the NFAT family, it is understood that the present invention may employ any one or a combination of these or other NFATs that are found in muscle cells.

In particular embodiments, the modulator of calcineurin activity inhibits the activity of an NFAT. More particularly, inhibition of the activity of an NFAT comprises inhibiting the dephosphorylation of NFAT. In other embodiments, the inhibition of the activity of an NFAT comprises reducing the expression of NFAT. In still further embodiments, inhibition of the activity of an NFAT comprises contacting NFAT with an agent that binds to and inactivates NFAT. In additional embodiments, inhibition of the activity of an NFAT comprises inhibiting the interaction of NFAT with MEF2. In specific embodiments, the agent that reduces the expression of an NFAT is an antisense construct. In other embodiments, the agent that binds to and inactivates an NFAT is an antibody or a small molecule inhibitor.

Also provided herein is a method of transforming a fast muscle fiber to a slow muscle fiber comprising increasing the calcineurin activity in the fast muscle fiber. More specific embodiments contemplate that the calcineurin is encapsulated in a liposome. In alternative embodiments, it is contemplated that the method of transforming a fast fiber to a slow fiber comprises the steps of providing an expression construct comprising a first nucleic acid encoding an active calcineurin and a promoter functional in the muscle fibers, wherein the nucleic acid is under transcriptional control of the promoter; and contacting the expression construct with the fast muscle fiber in an amount effective to promote the activation of NFAT of the fiber; wherein activation of NFAT in the fast fiber promotes the transformation of fast fiber to slow fiber.

In particularly contemplated embodiments, the fast fiber is located within an animal. In certain other embodiments, the first nucleic acid is a cDNA or genomic DNA. In certain preferred embodiments, the first expression construct is selected from the group consisting of an adenovirus, an adeno-associated virus, a vaccinia virus a herpes virus and a lentivirus. In other embodiments, the promoter may be selected from the group consisting of CMV IE, SV40 IE, RSV, β-actin, tetracycline regulatable and ecdysone regulatable. In particular embodiments, the contacting may be effected by direct injection of a muscle containing the slow fiber with the expression construct. In additional specific embodiments, the contacting may comprise delivering the expression construct intravenously, subcutaneously, intramuscularly, or intraperitoneally to a muscle containing the fast fiber.

Also contemplated herein is a method of transforming a slow muscle fiber to a fast muscle fiber comprising inhibiting calcineurin activity in the slow muscle fiber. More specifically, the method comprises the steps of providing an expression construct comprising a first nucleic acid encoding a calcineurin gene positioned antisense to a promoter functional in the slow muscle fiber, wherein the nucleic acid is under transcriptional control of the promoter; and contacting the expression construct with the slow muscle fiber in an amount effective to decrease the calcineurin activity in the fiber; wherein the decrease in calcineurin activity in the slow fiber promotes the transformation of slow fiber to fast fiber. In specific embodiments, it is contemplated that the method comprises contacting the slow muscle fiber with cyclosporin, FK506, AP1510 and FK1012. In other particular embodiments, the method comprises inhibiting the interaction of NFAT with MEF2.

Another aspect of the present invention provides a method of screening for modulators of muscle fiber phenotype comprising the steps of providing a skeletal muscle cell expressing an NFAT and/or a MEF2 gene; contacting the cell with a candidate modulator; and monitoring the cell for a phenotype that is absent when the cell is not treated with the candidate modulator. In preferred embodiments, the cell is in an animal. In other embodiments, the cell is derived from a fast muscle cell line. In yet another alternative, the cell is derived from a slow muscle cell line. It is contemplated that the contacting may be performed *in vitro*. In preferred embodiments, the monitoring may comprise measuring the activity or expression of a fast fiber-specific gene. In other embodiments, the monitoring may comprise measuring the activity or expression of a slow fiber-specific gene. In alternative embodiments, the monitoring may

comprise measuring the size or mass of the cell. In preferred embodiments, the monitoring may comprise monitoring Ca^{++} response in the cell. Specifically, monitoring the Ca^{++} response may comprise monitoring Ca^{2+} dependent gene expression in the cell. In certain embodiments, the contacting may be performed *in vivo*. In particular embodiments, the candidate modulator may be an antisense construct, a small molecule library, an antibody or more particularly a single chain antibody.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Response of different promoters to forced expression of a constitutively active form of calcineurin (O'Keefe *et al.*, 1992) in cultured C2C12 myotubes or NIH 3T3 fibroblasts. Promoter-reporter plasmids were constructed to link the indicated promoters (CMV, cytomegalovirus; TATA, a minimal promoter consisting of the TATA element from the human hsp70 gene; MCK, a 4.8 kb 5' flanking region from the murine muscle creatine kinase gene; Tnl slow, a 4.2 kb 5' flanking region from the human slow fiber-specific troponin I gene; or myoglobin, a 2 kb 5' flanking region from the human myoglobin gene) to a firefly luciferase reporter gene. The response to activated calcineurin was calculated as the fold-change in luciferase activity induced by activated calcineurin above that measured following transfection of the empty vector alone, corrected for transfection efficiency (β-galactosidase activity). Cyclosporin A (CsA) was added to the culture medium at the indicated final concentrations.

Histograms depict mean values (± SE) from 4-8 independent transfections in each cell background.

FIG. 2A and FIG. 2B. Role of NFAT proteins in calcineurin-dependent transactivation. Activity of wild-type and mutated myoglobin (FIG. 2A) or troponin I slow (FIG. 2B) gene promoters in differentiated C2C12 cells as a function of increasing doses of the calcineurin expression plasmid. Consensus NFAT recognition motifs at the indicated positions relative to the transcriptional start sites (see FIG. 6) were altered (ΔNFAT) by site-directed mutagenesis, and transfections were performed as described in FIG. 1. Data points represent mean values of luciferase activity, corrected for transfection efficiency (β-galactosidase activity), from duplicate transfections in a representative experiment, and expressed as a percentage of native promoter activity after transfection with the indicated amounts of activated calcineurin expression plasmid (CMV-CnA*).

FIG. 3A and FIG. 3B. Upstream regulatory elements of the myoglobin gene participating in calcineurin-dependent transactivation. Data are presented as reporter gene expression (mean ± SE of 6 independent transfections) normalized to activity of a cotranfected CMV-lacZ plasmid (luminometer units ($x10^5$)/well (1.9×10^5 cells)). (FIG. 3A) Responses of native (Mb380) or mutated variants of a truncated segment (-373 to +7) of the human myoglobin gene promoter to activated calcineurin. Nucleotide substitutions were introduced into each of two upstream regulatory elements shown previously to be essential for muscle-specific promoter activity (Devlin *et al.*, 1989; Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995; Grayson *et al.*, 1998). These mutated promoters (MbΔA/T and MbΔCCAC) are likewise defective for calcineurin-stimulated transactivation. (FIG. 3B) Responses to activated calcineurin of synthetic promoters constructed with various combinations of multimerized oligonucleotide cassettes representing protein binding motifs (CCAC, Sp1 binding site; A/T, MEF2 binding site; NRE, putative NFAT binding site; TATA, TBP binding site and core promoter) from the myoglobin promoter.

FIG. 4. Fiber composition of soleus muscles from intact rats treated with cyclosporin A. Circles represent individual animals (open, vehicle-treated; closed, cyclosporin A treated), and mean values in each group (± SE) are shown as horizontal lines. The difference in group means was highly significant ($p<.001$ by unpaired Student's t test).

FIG. 5. Model for a calcineurin-dependent pathway linking specific patterns of motor nerve activity to distinct programs of gene expression that establish phenotypic differences between slow and fast myofibers. MEF2 is shown to represent the requirement for collaboration between activated NFAT proteins and muscle-restricted transcription factors in slow fiber-specific gene transcription, but other proteins (not shown) also are likely to participate.

FIG. 6A and FIG. 6B. NFAT consensus binding sequences are present within transcriptional control regions previously shown to direct transcription selectively in slow-oxidative myofibers (Parsons *et al.*, 1993; Levitt *et al.*, 1995; Qin *et al.*, 1997). (FIG. 6A) Consensus NFAT binding motifs in myoglobin, troponin I slow (TnI slow), and sarcomeric mitochondrial creatine kinase (sMtCK) promoters. (FIG. 6B) Conserved sequence blocks (CAGG, CCAC, MEF2, and E box) common to a Slow fiber-specific Upstream Regulatory Element (SURE) from the rat troponin I slow gene and a Fast fiber-specific Intronic Regulatory Element (FIRE) from the quail troponin I fast gene (Nakayama *et al.*, 1996). A predicted NFAT response element (darkly shaded) overlapping with an E-Box (MyoD binding site) and adjacent to a MEF-2 binding site.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Fiber type of skeletal muscle plays a role in numerous disorders including progressive myonecrosis (Duchenne's muscular dystrophy) (Webster *et al.*, 1988), congestive heart failure, (Massie *et al.*, 1988; Sabbah *et al.*, 1993), decline in slow fibers observed as a result of prolonged inactivity or hypogravity (Caiozzo *et al.*, 1994), and the fiber composition of skeletal muscles influences insulin sensitivity (Kong *et al.*, 1994) and lipoprotein metabolism and atherosclerosis (Tikkanen *et al.*, 1996).

There is a well-established association between motor nerve activity and specialized fiber characteristics that has been described extensively in the literature on muscle plasticity (Michel *et al.*, 1994; Neufer *et al.*, 1996; Williams and Neufer, 1996). Fast-to-slow fiber transformation is evoked by increased motor nerve activity, stimulated by cross-innervation, electrical pacing or exercise training. Slow-to-fast fiber transformation occurs as a

consequence of decreased motor nerve activity, resulting from cross-innervation, certain disease states, hypogravity, or physical inactivity. The present invention describes methods and compositions for controlling these transformations.

1. The Present Invention

Slow- and fast-twitch myofibers of adult skeletal muscles express unique sets of muscle-specific genes, and these distinctive programs of gene expression are controlled by variations in motor neuron activity. It is well established that, as a consequence of more frequent neural stimulation, slow fibers maintain higher levels of intracellular free calcium than fast fibers, but the mechanisms by which calcium may function as a messenger linking nerve activity to changes in gene expression in skeletal muscle were unknown. Here, fiber type-specific gene expression in skeletal muscles is shown to be controlled by a signaling pathway that involves calcineurin, a cyclosporin-sensitive, calcium-regulated serine/threonine phosphatase. Activation of calcineurin in skeletal myocytes selectively up-regulates slow fiber-specific gene promoters. Conversely, inhibition of calcineurin activity by administration of cyclosporin A (CsA) to intact animals promotes slow-to-fast fiber transformation. Transcriptional activation of slow fiber-specific transcription appears to be mediated by a combinatorial mechanism involving proteins of the NFAT and MEF2 families. The present invention thus identifies a molecular mechanism by which different patterns of motor nerve activity promote selective changes in gene expression to establish the specialized characteristics of slow and fast myofibers.

The results described herein show a molecular model, not previously considered, to explain how motor nerve activity controls programs of gene expression that define fast and slow subtypes of skeletal myofibers (FIG. 5). The model proposes that tonic motor nerve activity, characteristic of nerves innervating slow muscles, sustains $[Ca^{2+}]_i$ at levels sufficient to activate the calcineurin-NFAT pathway. The protein phosphatase activity of calcineurin leads to dephosphorylation and nuclear localization of NFAT proteins. In the nucleus, NFAT proteins bind DNA in conjunction with other transcriptional regulators, including (but not limited to) MEF2, binding sites for which are clustered in promoter/enhancer regions controlling transcription of genes encoding proteins of the slow fiber program. In fast fibers, high amplitude calcium transients stimulated by infrequent, phasic firing of the motor nerve are of insufficient duration to maintain calcineurin in the active state, so NFAT proteins remain

phosphorylated and are excluded from the nucleus. When NFAT proteins are unavailable for DNA binding and protein-protein interactions at target promoters, the slow fiber-specific program is down-regulated, and genes encoding fast fiber-specific proteins are transcribed.

Using the insights gained herein, the present invention is directed to methods of altering the phenotype of skeletal muscle tissue, by contacting the tissue with a modulator of calcineurin activity. Thus, the method of the present invention may be used to increase the proportion of slow fiber to fast fiber in said tissue. Conversely, the methods may be used to increase of the proportion fast fiber to slow fiber in the tissue. Additional embodiments contemplate methods of screening for modulators of muscle fiber phenotype. These and other aspects of the present invention are discussed in further detail herein below.

2. A Calcineurin-Dependent Pathway in Muscle Fiber

It is known that $[Ca^{2+}]i$ levels play a role in the muscle fiber type characteristics of skeletal muscle. However, the possibility that calcineurin might be involved in skeletal muscle fiber type specialization has not been previously investigated. The present invention describes calcineurin-dependent transformation from fast to slow muscle fiber by transactivation of slow fiber-specific gene promoters, whereas inhibition calcineurin leads to a preponderance of fast fiber type phenotype. This pathway is depicted in FIG. 5. The individual components of this pathway, as they relate to muscle fiber composition in skeletal muscle, are discussed in further detail herein below.

a. Calcineurin

Calcineurin is a ubiquitously expressed serine/threonine phosphatase that exists as a heterodimer, comprised of a 59 kD calmodulin-binding catalytic A subunit and a 19 kD Ca^{2+}-binding regulatory B subunit (Stemmer and Klee, 1994; Su *et al.*, 1995). Calcineurin is activated by a sustained Ca^{2+} plateau and is insensitive to transient Ca^{2+} fluxes as occur in response (Dolmetsch *et al.*, 1997). Activation of calcineurin is mediated by binding of Ca^{2+} and calmodulin to the regulatory and catalytic subunits, respectively.

Calcineurin-dependent signaling mechanisms have been characterized extensively in the activation of cytokine gene expression in T and B lymphocytes responding to stimuli that elevate intracellular free calcium concentration ($[Ca^{2+}]_i$) (Rao *et al.*, 1997). Binding of calcium

to a calmodulin-calcineurin complex stimulates serine/threonine phosphatase activity of calcineurin, the major substrates of which are NFAT (Nuclear Factor of Activated T cells) transcription factors.

Dephosphorylation of NFATs by calcineurin promotes their translocation from the cytoplasm to the nucleus, where they bind a cognate nucleotide recognition sequence (Rao *et al.*, 1997) and stimulate transcription of target genes that, in lymphocytes, include hematopoietic growth factors (*e.g.*, GM-CSF) and inflammatory cytokines (*e.g.*, IL-2). Recent studies demonstrate that calcineurin activity and the resulting nuclear translocation of NFAT are insensitive to transient, high amplitude oscillations in $[Ca^{2+}]_i$ that activate other calcium-dependent events (*e.g.*, NF-κB or c-Jun N-terminal kinase). Rather, the calcineurin-NFAT pathway responds preferentially to sustained, low amplitude elevations of $[Ca^{2+}]_i$ (Timmerman *et al.*, 1996; Dolmetsch *et al.*, 1997). This ability of a calcineurin-dependent signaling pathway to discriminate between different patterns in the amplitude and duration of changes in $[Ca^{2+}]_i$, and the observation that there are differences in the $[Ca^{2+}]$ among specialized myofiber subtypes, provide the backdrop for the present invention.

The present invention demonstrates that forced expression of constitutively active calcineurin selectively transactivates promoters from two genes that are expressed preferentially in slow versus fast skeletal myofibers. Thus, downstream effectors of a calcineurin-regulated signaling pathway are present and capable of transducing the signal in a muscle cell background, and transcriptional regulatory elements capable of receiving the signal are present within genes representative of the slow fiber program. Specific effector molecules appear to include NFAT proteins, since consensus NFAT binding motifs contained within slow fiber-specific promoters participate in the response to activated calcineurin, and several NFAT isoforms are expressed in skeletal muscle (Hoey *et al.*, 1995).

Further, the data described herein show that DNA binding of NFAT proteins is not sufficient to transduce the calcineurin-generated signal in skeletal myocytes. Rather, NFAT transcription factors collaborate with MEF2 and other transcriptional regulatory proteins, the correct combination of which is present within differentiated myotubes, but absent from undifferentiated myoblasts or fibroblasts. Previous studies of calcineurin-stimulated transactivation of cytokine gene promoters in T cells, where AP-1 cooperates with NFAT in

both DNA binding and transactivation (reviewed by Rao *et al.*, 1997), provide a precedent for synergistic combinatorial interactions between NFAT proteins and heterologous transcription factors.

The organization of transcriptional control regions that confer fiber type-specific expression (FIG. 6) is consistent with this viewpoint. Consensus NFAT binding sequences are conserved in the 5' flanking region of myoglobin and TnIs genes from all vertebrate species in which promoter sequences are available, and in other slow fiber-specific enhancers (FIG. 6A). Moreover, two studies that have mapped fiber type-specific enhancers at the highest resolution. Buonanno and colleagues (Nakayama *et al.*, 1996) identified a 128-bp element from the rat TnIs gene (Slow Upstream Regulatory Element (SURE)) that confers slow fiber-specific transcription in transgenic mice, and a 144-bp element (Fast Intronic Regulatory Element (FIRE)) that directs fast fiber-specific expression of a different isoform of troponin I (TnIf).

The functionally distinctive SURE and FIRE elements contain similar or identical CAGG, CCAC, MEF2, and E box motifs (FIG. 6B), as found in many muscle-specific genes, so the basis for their reciprocal functions in specialized subtypes of myofibers has not been apparent. Examination of the SURE and FIRE elements in light of the observations described herein reveals a consensus NFAT recognition motif in the TnIs SURE element that is absent from the TnIf FIRE element. In the sarcomeric mitochondrial creatine kinase (sMtCK) gene, a 160-bp upstream element was shown to direct fiber type-specific gene expression in transgenic mice (Qin *et al.*, 1997). The sMtCK gene is expressed preferentially in slow, oxidative myofibers, in contrast to the MCK isoform (fast fiber specific) that was studied herein (FIG. 1). Like the myoglobin and TnIs gene enhancers, this sMtCK enhancer includes NFAT recognition motifs (FIG. 6A).

Furthermore, inhibition of calcineurin phosphatase activity in intact animals by administration of cyclosporin A leads to down-regulation of slow and induction of fast fiber type-specific markers. Methods and compositions for modulating this calcineurin-dependent effect in muscle fibers are presented herein below

b. Interaction between NFAT and Transcription Factors

Several forms of nuclear factor of activated T-cells (NFAT) are known to those of skill in the art, including NFAT1, NFAT2, NFATc, NFAT3, NFAT4a, NFAT4b and NFAT4c. Particular human NFATs are described in detail in U.S. Patent Number 5,708,158 (specifically incorporated herein by reference in its entirety). Additional information regarding the molecular interactions of NFAT proteins is provided by Nolan (1994). Northrop *et al.*, describes the cloning of a cDNA encoding human NFATc (1994). The cloning of a fragment of a gene encoding a murine NFAT1 also has been described (McCaffrey *et al.*, 1993)

These factors bind the consensus DNA sequence GGAAAAT as monomers or dimers through a Rel homology domain (RHD) (Rooney *et al.*, 1994; Hoey *et al.*, 1995). Three of the NFAT genes are restricted in their expression to T-cells and skeletal muscle, whereas NFAT3 is expressed in a variety of tissues including the heart (Hoey *et al.*, 1995). For additional disclosure regarding NFAT proteins the skilled artisan is referred to U. S. Patent 5,708,158.

Functional NFAT binding sites have been found in the promoters or enhancers of several different cytokine genes including IL-2, IL-4, IL-3, GM-CSF, and TNF-α and are often located next to AP-1 binding sites, which are recognized by members of the fos and jun families of transcription factors. Typically, the AP-1 binding sites adjacent to NFAT sites are low affinity sites, and AP-1 proteins cannot bind them independently. However, many NFAT and AP-1 protein combinations are capable of cooperatively binding to DNA. Furthermore, cell-type specificity of cytokine gene transcription is often controlled, at least in part, by the combinations of NFAT and AP-1 proteins present in those cells. For example, there are different classes of T cells that secrete different sets of cytokines: *e.g.*, TH1 cells produce IL-2 and IFN-γ, while TH2 cells produce IL-4, IL-5, and IL-6. NFAT binding sites are involved in the regulation of both TH1 and TH2 cytokines. Further, differential expression of the cytokine gene in T cell subsets is controlled the combinatorial interactions of NFAT and AP-1 proteins. Thus, NFAT has the ability to interact with additional factors in order to cooperatively bind DNA.

Recently, a calcineurin-dependent transcriptional pathway was shown to promote hypertrophic growth of the heart (Molkentin *et al.*, 1998). In cardiac myocytes, this pathway was shown to involve collaborative interactions between activated NFAT proteins and GATA4,

a cardiac-restricted transcription factor not present in skeletal muscle. Cardiomyocytes express several isoforms of MEF2, and many of the same genes that exhibit slow, oxidative fiber type-specific expression in skeletal muscle are transcriptionally active in cardiac myocytes (*e.g.*, myoglobin or sMtCK (Parsons *et al.*, 1993; Levitt *et al.*, 1995; Qin *et al.*, 1997).

Given the ability of NFAT factors to mediate changes in gene expression in response to Ca^{2+} signaling in T cells, and of cardiac gene expression, the inventors results presented herein suggest a mechanism for coupling calcineurin signaling to muscle fiber specific gene transcription.

c. Inhibitors of Calcineurin

Cyclosporin (CsA) and FK-506, bind the immunophilins cyclophilin and FK-506-binding protein (FKBP12), respectively, forming complexes that bind the calcineurin catalytic subunit and inhibit its activity. The results presented herein show that CsA and FK-506 inhibit calcineurin activity in intact animals and this block in calcineurin activity promotes slow-to-fast fiber transformation. Of note, it is known that hypertrophic agonists in heart muscle act by elevating intracellular Ca^{++}, which results in activation of the PKC and MAP kinase signaling pathways (Sadoshima and Izumo, 1993a, 1993b; Kudoh *et al.*, 1997; Yamazaki *et al.*, 1997; Zou *et al.*, 1996). CsA does not interfere with early signaling events at the cell membrane, such as PI turnover, Ca^{++} mobilization, or PKC activation (Emmel *et al.*, 1989).

In the context of the present invention, inhibitors of calcineurin activity have been shown to promote the transition between slow twitch fiber to fast fiber phenotype. This important observation answers why pharmacological blockade of calcineurin signaling in rats reduces the capacity for endurance exercise and diminishes peak rates of oxidative phosphorylation in mitochondria isolated from skeletal muscles (Mercier *et al.*, 1995). Clearly, the present results have a significant clinical relevance, revealing a need to modify skeletal muscle physiology and exercise performance in patients receiving CsA and FK506 and derivatives thereof in immunosuppressant drugs in current clinical use.

In specific embodiments of the present invention, it may be desirable to increase the proportion of fast fiber content in a slow muscle fiber population. Given the teachings of the present invention, such an effect may be achieved by the application of CsA, FK506, AP1510,

FK1012, any derivative thereof or any additional immunosuppressant that acts by inhibiting calcineurin expression, activity or function.

d. Muscle Fiber Type-Specific Genes

The present invention provides methods and compositions for altering the phenotype of a particular muscle fiber. The phenotypic properties of slow- and fast-twitch myofibers are determined by the selective transcription of genes coding for contractile proteins and metabolic enzymes in these muscles. Genes associated with fast fibers include, but are not limited to, muscle creatinine kinase, fast myosin heavy chain, (Hoh, 1992), fast myosin light chain (Jostarndt *et al.*, 1996; Rao *et al.*, 1996), fast troponin (Nakayama *et al.*, 1996; Briggs and Schachat, 1996) and parvalbumin (Nishida *et al.*, 1997). Genes associated with slow fibers include but are not limited to myoglobin (Williams *et al.*, 1997; Shen *et al.*, 1996), troponin I (Matsumoto *et al.*, 1997; Nakayama *et al.*, 1996; Levitt *et al.*, 1995), slow myosin heavy chain (Hoh, 1992; Mair *et al.*, 1992), slow myosin light chain (Jostarndt *et al.*,1996), mitochondrial proteins (Howlett and Willis, 1998; Delp *et al.*, 1997; Ogata and Yamasaki, 1997; Nakano *et al.*, 1997) GLUT4 (Marette *et al.*, 1992; Kong *et al.*, 1994; Ivy, 1996), or lipoprotein lipase (Borensztajn *et al.*, 1975; Kaciuba-Uscilko *et al.*, 1980; Mackie *et al.*, 1980; Tikkanen *et al.*, 1996). The genes specific for the slow muscle fiber phenotype possess binding sites for NFAT. Additionally, these genes may possess binding sites for transcription factors such as MEF2 and the like. The references above (each specifically incorporated herein by reference) describe the protein compositions of slow and fast twitch muscles. However, it is understood that these are exemplary in nature, additional fiber specific genes are well known to those of skill in the art and are contemplated for use in conjunction with the present invention.

3. Modulating Fiber Type Composition

Although the connection between $[Ca^{2+}]i$ and muscle tonic activity is well established, the present invention provides the first evidence of a calcineurin mediated pathway for the specialization of muscle fiber type. Essentially, the calcineurin is found to activate cytoplasmic NFAT by dephosphorylation. The dephosphorylated NFAT is translocated into the nucleus where it interacts with MEF2 and/or other transcription factors and upregulates the genes specific for slow fiber type muscle (*e.g.*, myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase).

Thus, in a particular embodiment of the present invention, there are provided methods of altering the phenotype of skeletal muscle tissue, by contacting the tissue with a modulator of calcineurin activity. These methods exploit the inventors' observation, described in detail herein, that calcinuerin, presumably through the involvement of one or more NFAT proteins, appears to up-regulate the expression of genes involved in the slow fiber muscle type, whereas inhibition of calcineurin up-regulates the expression of fast muscle fiber type.

At its most basic, the modulator of calcineurin activity will increase or potentiate the effect of calcineurin. This will function by increasing the *in vivo* activity of NFAT in the muscle fiber of an individual in need of slow muscle fiber type. Such a modulator will be useful in conditions presenting progressive myonecrosis, *e.g.*, Duchenne's muscular dystrophy and other diseases characterized by myodegeneration, myonecrosis and the like. This may be accomplished by one of several different mechanisms. First, one may increase the expression of calcineurin. Second, one may directly increase the function of the calcineurin protein by providing an agent activates the calcineurin protein, *e.g.*, calmodulin, Ca^{2+} and the like. And third, one may indirectly potentiate the effect of calcineurin by increasing the activity or expression of with one or more the NFAT targets of calcineurin. Alternatively, the effect may be further downstream of NFAT in which the activity of a transcription factor, such as a MEF2 or a gene influenced by the interaction of MEF2 and NFAT, such as myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase is increased.

In other embodiments, it may prove useful to increase the proportion of fast fiber in a particular muscle fiber. This embodiment will entail the inhibition of calcineurin activity. Such inhibition may be achieved by blocking the expression of calcineurin and/or the substrate for calcineurin activity (*i.e.*, NFAT). A second alternative would be to contact the muscle tissue with a one may directly decrease the function of the calcineurin protein by providing an agent inactivates the calcineurin protein, *e.g.*, cyclosporin A, FK506 and the like.

a. Increasing Slow Fiber Proportion

It has been demonstrated herein that in skeletal muscle fibers, the presence of an activated calcineurin dephosphorylates NFAT which, in turn, translocates to the nucleus where the dephosphorylated NFAT interacts with MEF2. The NFAT/MEF2 complex activates the

transcription of slow fiber specific genes including, but not limited to, myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase. Thus, it is envisioned that the phenotype of a particular muscle fiber may be altered by providing to the muscle fiber an activated form of calcineurin.

In other embodiments, an approach to alter the phenotype simply involves is the provision of a calcineurin polypeptide, active fragments, synthetic peptides, mimetics or other analogs thereof. The protein may be produced by recombinant expression means or, if small enough, generated by an automated peptide synthesizer. Formulations would be selected based on the route of administration and purpose including, but not limited to, liposomal formulations and classic pharmaceutical preparations.

In yet another alternative, the present invention may be used to alter the phenotype of a fast muscle fiber cell by providing to the cell a stimulator of calcineurin activity. Stimulators of calcineurin include Ca^{2+} and calmodulin. Further, the present invention provides details of method of identifying additional modulators of muscle fiber phenotype herein below.

As the present invention shows that calcineurin dephosphorylates NFAT in muscle fibers, and it is the dephosphorylated NFAT that mediates the effects of calcineurin and promotes the specialization of muscle fibers into slow fibers, it may prove useful to provide dephosphorylated NFAT to a muscle fiber. The NFAT may be provided as a protein composition or may alternatively be provided as an expression construct as described herein below.

b. Increasing Fast Fiber Proportion

In order to increase the proportion of fast fiber content in a particular composition, it will be necessary to block, inhibit or otherwise abrogate the action of calcineurin. Methods for this embodiment of the invention are outlined herein below.

i. Blocking Expression of Calcineurin and/or NFAT

The most direct method for blocking protein expression is via antisense technology. The term "antisense" is intended to refer to polynucleotide molecules complementary to a portion of a given RNA (*e.g.*, calcineurin, NFAT1, NFAT2, NFAT3, NFAT4) or the DNAs corresponding

thereto. "Complementary" polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs for the present invention will include regions complementary to the mRNA start site. One can readily test such constructs simply by testing the constructs *in vitro* to determine whether levels of the target protein are affected. Similarly, detrimental non-specific inhibition of protein synthesis also can be measured by determining target cell viability *in vitro*.

As used herein, the terms "complementary" or "antisense" mean polynucleotides that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen nucleotides out of fifteen. Naturally, sequences which are "completely complementary" will be sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a

non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

The polynucleotides according to the present invention may encode a calcineurin or NFAT gene or a portion of those genes that is sufficient to effect antisense inhibition of protein expression. The polynucleotides may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In other embodiments, however, the polynucleotides may be complementary DNA (cDNA). cDNA is DNA prepared using messenger RNA (mRNA) as template. Thus, a cDNA does not contain any interrupted coding sequences and usually contains almost exclusively the coding region(s) for the corresponding protein. In other embodiments, the antisense polynucleotide may be produced synthetically.

It may be advantageous to combine portions of the genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

The DNA and protein sequences for human NFAT family members have been published and are disclosed in U.S. Patent 5 ,708,158. It is contemplated that natural variants of exist that have different sequences than those disclosed herein. Thus, the present invention is not limited to use of the provided polynucleotide sequence for calcineurin or NFAT but, rather, includes use of any naturally-occurring variants. Depending on the particular sequence of such variants, they may provide additional advantages in terms of target selectivity, *i.e.*, avoid unwanted antisense inhibition of related transcripts. The present invention also encompasses chemically synthesized mutants of these sequences.

Although the antisense sequences may be full length genomic or cDNA copies, or large fragments thereof, they also may be shorter fragments, or "oligonucleotides," defined herein as polynucleotides of 50 or less bases. Although shorter oligomers (8-20) are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of base-pairing. For example, both binding affinity and sequence specificity of an oligonucleotide to its complementary target increase with increasing length. It is contemplated that oligonucleotides

of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50 base pairs will be used. While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression.

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" is refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in both DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression vector encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense polynucleotide. Ribozyme sequences also may be modified in much the same way as described for antisense polynucleotide. For example, one could incorporate non-Watson-Crick bases, or make mixed RNA/DNA oligonucleotides, or modify the phosphodiester backbone, or modify the 2'-hydroxy in the ribose sugar group of the RNA.

Alternatively, the antisense oligo- and polynucleotides according to the present invention may be provided as RNA via transcription from expression constructs that carry nucleic acids encoding the oligo- or polynucleotides. Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid encoding an antisense product in which part or all of the nucleic acid sequence is capable of being transcribed. Typical expression vectors include bacterial plasmids or phage, such as any of the pUC or Bluescript™ plasmid series or, as discussed further below, viral vectors adapted for use in eukaryotic cells.

In preferred embodiments, the nucleic acid encodes an antisense oligo- or polynucleotide is placed in a replicable cloning vehicle that supports expression of the antisense molecule with

cis-acting transcriptional and translational signals. The expression constructs will comprise the gene in question and various regulatory elements as described herein below.

ii. Blocking Function of Calcineurin

In another embodiment, it may be desirable to block the function of a calcineurin and/or NFAT polypeptide rather than inhibit expression. This can be accomplished by use of organochemical compositions that interfere with the function of the protein, by use of an antibody that blocks an active site or binding site on calcinuerin or NFAT, or by use of a molecule that mimics a target of calcineurin (*i.e.*, NFAT) or of NFAT (*i.e.*, MEF2, GATA4 and the like).

With respect to organochemical inhibitors, such compounds may be identified in standard screening assays. For example, it is known that calcineurin bind to NFAT. Various candidate substances can be contacted with calcineurin followed by further determination of the ability of treated calcineurin to bind NFAT. Alternatively, given the knowledge that NFAT is activated as a result of dephosphorylation by calcineurin, and it is this activation that produces the upregulation of the slow fiber phenotype, it now is possible to provide an activator or inhibitor *in vivo* to an appropriate animal, *e.g.*, a mouse, and look for decreased muscle growth. Once identified, such a modulator may be used to stimulate or inhibit calcineurin and/or NFAT3 function in a therapeutic context.

With respect to antibodies, it should be noted that not all antibodies are expected to have the same functional effects on their targets. This stems both from the differing specificities of antibodies and their character, *i.e.*, their isotype. Thus, it will be useful to generate a number of different monoclonal and polyclonal preparations against calcineurin. It also may prove useful to generate anti-idiotypic antibodies to anti-calcineurin antibodies. These compounds may be used as probes for calcineurin binding partners such as members of the NFAT family. Additional similar antibodies directed against NFAT may be used to identify NFAT putative binding partners, such as MEF2 and other nuclear transcriptional factors.

The methods by which antibodies are generated are well known to those of skill in the art, and are detailed elsewhere in the specification. Again, antibodies that bind to calcineurin

may be screened for other functional attributes, *e.g.*, blocking of NFAT binding, blocking of dephosphorylation of NFAT *etc.* in *in vitro* assays prior to their implementation *in vivo*.

A particularly useful antibody for blocking the action of a given protein is a single chain antibody. Methods for the production of single-chain antibodies are well known to those of skill in the art. The skilled artisan is referred to U.S. Patent 5,359,046, (incorporated herein by reference) for such methods. A single chain antibody, preferred for the present invention, is created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (Fvs) in which the C-terminus of one variable domain is tethered to the N-terminus of the other via a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk *et al.*, 1990; Chaudhary *et al.*, 1990). These Fvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody.

With respect to inhibitors that mimic calcineurin or NFAT targets, the use of mimetics provides one example of custom designed molecules. Such molecules may be small molecules that specifically stimulate or inhibit calcineurin or NFAT protein activity or alternatively, stimulate or inhibit binding to MEF2. Such molecules may be sterically similar to the actual target compounds, at least in key portions of the target's structure and or organochemical in structure. Alternatively, these inhibitors may be peptidyl compounds, these are called peptidomimetics. Peptide mimetics are peptide-containing molecules which mimic elements of protein secondary structure. See, for example, Johnson *et al.*, (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of ligand and receptor. An exemplary peptide mimetic of the present invention would, when administered to a subject, bind to NFAT in a manner analogous to MEF2.

Successful applications of the peptide mimetic concept have thus, far focused on mimetics of β-turns within proteins, which are known to be highly antigenic. Likely β-turn structures within an antigen of the invention can be predicted by computer-based algorithms as discussed above. Once the component amino acids of the turn are determined, mimetics can be constructed to

achieve a similar spatial orientation of the essential elements of the amino acid side chains, as discussed in Johnson *et al.*, (1993).

iii. Blocking of a Target

As discussed above, one of the benefits of the present invention is the identification of targets upon which calcineurin acts. These targets may be binding partners such as NFAT. The NFAT in turn binds to MEF2 or other genes that are upregulated by an activated NFAT interaction with MEF2, such as myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase. In order to prevent calcineurin from interacting with these targets, one may take a variety of different approaches. For example, one may generate antibodies against the target and then provide the antibodies to the subject in question, thereby blocking access of calcineurin to the target molecule. Equally antibodies against NFAT will prevent the binding of NFAT to MEF2 and therefore prevent the upregulation of slow twitch specific genes.

In yet another embodiment, antisense methodologies may be employed in order to inhibit the interaction of NFAT with its target, seeing as the NFAT binding partner is a DNA molecule. Alternatively, one may design a polypeptide or peptide mimetic that is capable of interacting with the NFAT target in the same fashion as NFAT, but without any NFAT-like effect on the target.

In a preferred embodiment, the present invention will provide an agent that binds competitively to MEF2. In a more preferred embodiment, the agent will have an even greater affinity for the MEF2 than does NFAT does. Affinity for the MEF2 can be determined *in vitro* by performing kinetic studies on binding rates.

Other compounds may be developed based on computer modeling and predicted higher order structure, both of the calcineurin, NFAT and of the identified NFAT target molecules. This approach has proved successful in developing inhibitors for a number of receptor-ligand interactions.

4. Genetic Constructs and Gene Transfer

In particular embodiments, it will be desirable to place a calcineurin or an NFAT gene into expression constructs and monitor their effect on the muscle fiber specific gene expression. For example, calcineurin or NFAT may be tested by introducing into cultured muscle fiber cells an expression construct comprising a promoter operably linked to the calcineurin or NFAT gene or genes and monitoring the expression of the genes. A slow muscle fiber specific effect may be demonstrated when there is an increase in expression of slow muscle fiber specific genes.

Expression constructs also are used in generating transgenic animals, such constructs have a promoter for expression of the construct in an animal cell and a region encoding a gene product which modulates transcription of at least one gene that is expressed in myocytes in response to a particular signal responsible for fiber specific expression. In other embodiments, the expression construct encodes an antisense oligo- or polynucleotide is placed in a replicable cloning vehicle that supports expression of the antisense molecule for the therapeutic purposes discussed above.

a. Genetic Constructs

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for gene products in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding genes of interest.

i. Promoters

Transcriptional regulatory elements which are suitable for use in the present invention include which direct the transcription of a coding region to which they are operably linked preferentially in myocytes. By "preferentially" is meant that the expression of the transgene in myocytes is at least about 10-fold, more preferably at least about 10-fold to about 50-fold, even more preferably at least about 50-fold to 100-fold, even more preferably more than 100-fold greater than that in non-myocytes. Preferably, expression of the transgene is below detectable

limits in cells other than myocytes, as indicated by reporter gene assays well known to those of skill in the art.

The nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, β-actin, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. For example in the case where expression of a transgene, or transgenes when a multicistronic vector is utilized, is toxic to the cells in which the vector is produced in, it may be desirable to prohibit or reduce expression of one or more of the transgenes. Examples of transgenes that may be toxic to the producer cell line are pro-apoptotic and cytokine genes. Several inducible promoter systems are available for production of viral vectors where the transgene product may be toxic.

The ecdysone system (Invitrogen, Carlsbad, CA) is one such system. This system is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility. The system is based on the heterodimeric ecdysone receptor of Drosophila, and when ecdysone or an analog such as muristerone A binds to the receptor, the receptor activates a promoter to turn on expression of the downstream transgene high levels of mRNA transcripts are attained. In this system, both monomers of the heterodimeric receptor are constitutively expressed from one vector, whereas the ecdysone-

responsive promoter which drives expression of the gene of interest is on another plasmid. Engineering of this type of system into the gene transfer vector of interest would therefore be useful. Cotransfection of plasmids containing the gene of interest and the receptor monomers in the producer cell line would then allow for the production of the gene transfer vector without expression of a potentially toxic transgene. At the appropriate time, expression of the transgene could be activated with ecdysone or muristeron A.

Another inducible system that would be useful is the Tet-Off™ or Tet-On™ system (Clontech, Palo Alto, CA) originally developed by Gossen and Bujard (Gossen and Bujard, 1992; Gossen *et al.*, 1995). This system also allows high levels of gene expression to be regulated in response to tetracycline or tetracycline derivatives such as doxycycline. In the Tet-On™ system, gene expression is turned on in the presence of doxycycline, whereas in the Tet-Off™ system, gene expression is turned on in the absence of doxycycline. These systems are based on two regulatory elements derived from the tetracycline resistance operon of *E. coli*. The tetracycline operator sequence to which the tetracycline repressor binds, and the tetracycline repressor protein. The gene of interest is cloned into a plasmid behind a promoter that has tetracycline-responsive elements present in it. A second plasmid contains a regulatory element called the tetracycline-controlled transactivator, which is composed, in the Tet-Off™ system, of the VP16 domain from the herpes simplex virus and the wild-type tertracycline repressor. Thus, in the absence of doxycycline, transcription is constitutively on. In the Tet-On™ system, the tetracycline repressor is not wild-type and in the presence of doxycycline activates transcription. For gene transfer vector production, the Tet-Off™ system would be preferable so that the producer cells could be grown in the presence of tetracycline or doxycycline and prevent expression of a potentially toxic transgene, but when the vector is introduced to the patient, the gene expression would be constitutively on.

In some circumstances, it may be desirable to regulate expression of a transgene in a gene transfer vector. For example, different viral promoters with varying strengths of activity may be utilized depending on the level of expression desired. In mammalian cells, the CMV immediate early promoter if often used to provide strong transcriptional activation. Modified versions of the CMV promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. When expression of a transgene in hematopoetic cells is desired, retroviral promoters such as the LTRs from MLV or MMTV are often used. Other

viral promoters that may be used depending on the desired effect include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters such as from the E1A, E2A, or MLP region, AAV LTR, cauliflower mosaic virus, HSV-TK, and avian sarcoma virus.

Similarly tissue specific promoters may be used to effect transcription in specific tissues or cells so as to reduce potential toxicity or undesirable effects to non-targeted tissues. For example, promoters such as the PSA, probasin, prostatic acid phosphatase or prostate-specific glandular kallikrein (hK2) may be used to target gene expression in the prostate. Similarly, the following promoters may be used to target gene expression in other tissues.

It is envisioned that any of the above promoters alone or in combination with another may be useful according to the present invention depending on the action desired. In addition, this list of promoters is should not be construed to be exhaustive or limiting, those of skill in the art will know of other promoters that may be used in conjunction with the promoters and methods disclosed herein.

ii. Enhancers

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells *via* receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and

Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kB of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

iii. Polyadenylation Signals

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human or bovine growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

b. Gene Transfer

There are a number of ways in which expression vectors may introduced into cells. In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. In other embodiments, non-viral delivery is contemplated. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). Delivery mechanisms are discussed in further detail herein below.

i. Non-viral transfer

The present section provides a discussion of methods and compositions of non-viral gene transfer. DNA constructs of the present invention are generally delivered to a cell, and in certain situations, the nucleic acid or the protein to be transferred may be transferred using non-viral methods.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988).

Once the construct has been delivered into the cell the nucleic acid encoding the particular gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In another particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline condensed globules (Radler *et al.*, 1997). These DNA-lipid complexes are potential non-viral vectors for use in gene delivery.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Using the β-lactamase gene, Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection. Also included are various commercial approaches involving "lipofection" technology.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

Other vector delivery systems which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.*, (1987) employed lactosyl-ceramide, a galactose-terminal

asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky *et al.*, (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner *in vivo* and express CAM.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene application refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

ii. Viral Transfer

Adenovirus. One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct

and (b) to express an antisense polynucleotide, a protein, a polynucleotide (*e.g.*, ribozyme, or an mRNA) that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retroviruses, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. As used herein, the term "genotoxicity" refers to permanent inheritable host cell genetic alteration. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification of normal derivatives. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

The E3 region encodes proteins that appears to be necessary for efficient lysis of Ad infected cells as well as preventing TNF-mediated cytolysis and CTL mediated lysis of infected cells. In general, the E4 region encodes is believed to encode seven proteins, some of which

activate the E2 promoter. It has been shown to block host mRNA transport and enhance transport of viral RNA to cytoplasm. Further the E4 product is in part responsible for the decrease in early gene expression seen late in infection. E4 also inhibits E1A and E4 (but not E1B) expression during lytic growth. Some E4 proteins are necessary for efficient DNA replication however the mechanism for this involvement is unknown. E4 is also involved in post-transcriptional events in viral late gene expression; *i.e.*, alternative splicing of the tripartite leader in lytic growth. Nevertheless, E4 functions are not absolutely required for DNA replication but their lack will delay replication. Other functions include negative regulation of viral DNA synthesis, induction of sub-nuclear reorganization normally seen during adenovirus infection, and other functions that are necessary for viral replication, late viral mRNA accumulation, and host cell transcriptional shut off.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993; Shenk, 1978).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey

embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.*, (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking is initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical, medical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.*, (1986), or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression investigations (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene transfer (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993), intranasal inoculation (Ginsberg *et al.*, 1991), aerosol administration to lung (Bellon, 1996) intra-peritoneal administration (Song *et al.*, 1997), Intra-pleural injection (Elshami *et al.*, 1996) administration to the bladder using intra-vesicular administration (Werthman, *et al.*, 1996), Subcutaneous injection including intraperitoneal, intrapleural, intramuscular or subcutaneously) (Ogawa, 1989) ventricular injection into myocardium (heart, French *et al.*, 1994), liver perfusion (hepatic artery or portal vein, Shiraishi *et al.*, 1997) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

Retrovirus. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, *gag*, *pol*, and *env* that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol*, and *env* genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux

et al., 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

Herpesvirus. Because herpes simplex virus (HSV) is neurotropic, it has generated considerable interest in treating nervous system disorders. Moreover, the ability of HSV to establish latent infections in non-dividing neuronal cells without integrating in to the host cell chromosome or otherwise altering the host cell's metabolism, along with the existence of a promoter that is active during latency makes HSV an attractive vector. And though much attention has focused on the neurotropic applications of HSV, this vector also can be exploited for other tissues given its wide host range.

Another factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (temporal, strength, etc.) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations.

HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a

lessened need for repeat dosings. For a review of HSV as a gene transfer vector, see Glorioso *et al.*, (1995).

HSV, designated with subtypes 1 and 2, are enveloped viruses that are among the most common infectious agents encountered by humans, infecting millions of human subjects worldwide. The large, complex, double-stranded DNA genome encodes for dozens of different gene products, some of which derive from spliced transcripts. In addition to virion and envelope structural components, the virus encodes numerous other proteins including a protease, a ribonucleotides reductase, a DNA polymerase, a ssDNA binding protein, a helicase/primase, a DNA dependent ATPase, a dUTPase and others.

HSV genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974; Honess and Roizman 1975; Roizman and Sears, 1995). The expression of α genes, the first set of genes to be expressed after infection, is enhanced by the virion protein number 16, or α-transducing factor (Post *et al.*, 1981; Batterson and Roizman, 1983). The expression of β genes requires functional α gene products, most notably ICP4, which is encoded by the α4 gene (DeLuca *et al.*, 1985). γ genes, a heterogeneous group of genes encoding largely virion structural proteins, require the onset of viral DNA synthesis for optimal expression (Holland *et al.*, 1980).

In line with the complexity of the genome, the life cycle of HSV is quite involved. In addition to the lytic cycle, which results in synthesis of virus particles and, eventually, cell death, the virus has the capability to enter a latent state in which the genome is maintained in neural ganglia until some as of yet undefined signal triggers a recurrence of the lytic cycle. Avirulent variants of HSV have been developed and are readily available for use in gene transfer contexts (U.S. Patent 5,672,344).

Adeno-Associated Virus. Recently, adeno-associated virus (AAV) has emerged as a potential alternative to the more commonly used retroviral and adenoviral vectors. While studies with retroviral and adenoviral mediated gene transfer raise concerns over potential oncogenic properties of the former, and immunogenic problems associated with the latter, AAV has not been associated with any such pathological indications.

In addition, AAV possesses several unique features that make it more desirable than the other vectors. Unlike retroviruses, AAV can infect non-dividing cells; wild-type AAV has been characterized by integration, in a site-specific manner, into chromosome 19 of human cells (Kotin and Berns, 1989; Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991); and AAV also possesses anti-oncogenic properties (Ostrove *et al.*, 1981; Berns and Giraud, 1996). Recombinant AAV genomes are constructed by molecularly cloning DNA sequences of interest between the AAV ITRs, eliminating the entire coding sequences of the wild-type AAV genome. The AAV vectors thus, produced lack any of the coding sequences of wild-type AAV, yet retain the property of stable chromosomal integration and expression of the recombinant genes upon transduction both *in vitro* and *in vivo* (Berns, 1990; Berns and Bohensky, 1987; Bertran *et al.*, 1996; Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997a). Until recently, AAV was believed to infect almost all cell types, and even cross species barriers. However, it now has been determined that AAV infection is receptor-mediated (Ponnazhagan *et al.*, 1996; Mizukami *et al.*, 1996).

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription. The sequence of AAV is provided by Srivastava *et al.*, (1983), and in U.S. Patent 5,252,479 (entire text of which is specifically incorporated herein by reference).

The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-structural proteins apparently are derived from the longer of the transcripts, and three virion proteins all arise from the smallest transcript.

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of

the helpers is adenovirus, and many "early" functions for this virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

Vaccinia Virus. Vaccinia virus vectors have been used extensively because of the ease of their construction, relatively high levels of expression obtained, wide host range and large capacity for carrying DNA. Vaccinia contains a linear, double-stranded DNA genome of about 186 kb that exhibits a marked "A-T" preference. Inverted terminal repeats of about 10.5 kb flank the genome. The majority of essential genes appear to map within the central region, which is most highly conserved among poxviruses. Estimated open reading frames in vaccinia virus number from 150 to 200. Although both strands are coding, extensive overlap of reading frames is not common.

At least 25 kb can be inserted into the vaccinia virus genome (Smith and Moss, 1983). Prototypical vaccinia vectors contain transgenes inserted into the viral thymidine kinase gene *via* homologous recombination. Vectors are selected on the basis of a tk-phenotype. Inclusion of the untranslated leader sequence of encephalomyocarditis virus, the level of expression is higher than that of conventional vectors, with the transgenes accumulating at 10% or more of the infected cell's protein in 24 h (Elroy-Stein *et al.*, 1989).

Lentiviruses. Lentiviruses can also be used as vectors in the present application. In addition to the long-term expression of the transgene provided by all retroviral vectors, lentiviruses present the opportunity to transduce nondividing cells and potentially achieve regulated expression. The development of lentiviral vectors requires the design of transfer vectors to ferry the transgene with efficient encapsidation of the transgene RNA and with full expression capability, and of a packaging vector to provide packaging machinery *in trans* but without helper virus production. For both vectors, a knowledge of packaging signal is required- the signal to be included in the transfer vector but excluded from the packaging vector. Exemplary human lentiviruses are human immunodeficiency virus type 1 and type 2 (HIV-1 and HIV-2). HIV-2 is likely better suited for gene transfer than HIV-1 as it is less pathogenic and thus safer during design and production; its desirable nuclear import and undesirable cell-cycle arrest functions are segregated on two separate genes (Arya *et al.*, 1998; Blomer *et al.*, 1997).

c. Selection Methods

Primary mammalian cell cultures may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

One embodiment of the foregoing involves the use of gene transfer to immortalize cells for the production of proteins. The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. The generation of recombinant expression vectors, and the elements included therein, are discussed above. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell in question.

Examples of useful mammalian host cell lines are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, NIH3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and process the gene product in the manner desired. Such modifications

(*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the foreign protein expressed.

Thus, following introduction of the expression construct into the cells, expression of the reporter gene can be determined by conventional means. Any assay which detects a product of the reporter gene, either by directly detecting the protein encoded by the reporter gene or by detecting an enzymatic product of a reporter gene-encoded enzyme, is suitable for use in the present invention. Assays include colorimetric, fluorimetric, or luminescent assays or even, in the case of protein tags, radioimmunoassays or other immunological assays. Transfection efficiency can be monitored by co-transfecting an expression construct comprising a constitutively active promoter operably linked to a reporter gene.

A number of selection systems may be used including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk-*, *hgprt-* or *aprt-* cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to *gpt*; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

Animal cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

5. Screening For Modulators Of Muscle Fiber Composition

The present invention also contemplates the screening of compounds for their ability to modulate the composition of fibers of a particular muscle. The ability to create cellular, organ and organismal systems which mimmic the effects of a constitutively activated calcineurin on muscle cells provides an ideal setting in which to test various compounds for therapeutic activity. Particularly preferred compounds will be those useful in modulating the calcineurin

levels in the particular muscle and therefore modulating muscle fiber composition. In the screening assays of the present invention, the candidate substance may first be screened for basic biochemical activity -- *e.g.*, binding to a target molecule -- and then tested for its ability to modulate calcineurin activity, at the cellular, tissue or whole animal level.

a. Modulators and Assay Formats

i. Assay Formations

The present invention provides methods of screening for modulators of muscle fiber phenotype. It is contemplated that these screening techniques will prove useful in the identification of compounds that alter the phenotype of a muscle fiber from fast to slow or vice verse. It is contemplated that the modulator will achieve this effect by acting on the calcineurin pathway in the muscle cells

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to alter muscle fiber phenotype, generally including the steps of:

(a) providing a skeletal muscle cell expressing an NFAT and/or a MEF2 gene;

(b) contacting said cell with a candidate modulator; and

(c) monitoring said cell for a phenotype that is absent when said cell is not treated with said candidate modulator.

To identify a candidate substance as being capable of altering the phenotype of the muscle cell in the assay above, one would measure or determine various characteristics of the cell, for example, calcineurin activity, growth, Ca^{2+}-dependent gene expression and the like in the absence of the added candidate substance. One would then add the candidate substance to the cell and determine the response in the presence of the candidate substance. A candidate substance which increases stimulates or otherwise potentiates calcineurin activity or function is indicative of said modulator being a stimulator of slow fiber formation whereas a modulator which decreases inhibits or otherwise abrogate calcineurin activity or function is indicative of said modulator being a stimulator of fast fiber formation. In the screening assays of the present invention, the compound is added to the cells, over period of time and in various dosages, and the selected phenotype is measured.

In particularly preferred aspects, the cells express an NFAT and/or a MEF2 gene. In certain embodiments, the other genes involved in the NFAT pathway may be altered to achieve the same effect, such as a mutant form of MEF2 that is capable of functioning without the assistance of NFAT. It also is contemplated that the cells may express a fiber-specific gene having a promoter containing MEF2 and NFAT binding sites.

ii. Inhibitors and Activators of Calcineurin

An activator or stimulator of calcineurin activity according to the present invention may be one which exerts its activatory effect upstream or downstream of calcineurin, or on calcineurin directly. Regardless of the type of activator identified by the present screening methods, the effect of such a compound results in activation of genes that are responsible for slow twitch fiber phenotype and therefore leads to an increase in the presence of slow fiber phenotype over that of a fast fiber phenotype. In certain embodiments, a downstream signaling element may be installed into the cell such that an increase in a signal would indicate an increase in activity in the pathway. One conceivable signal would be a gene such as green fluorescent protein linked to a regulatory control region that was activated by NFAT/MEF2.

In alternative embodiments, the present invention provides methods for identifying an inhibitor of the calcineurin pathway in muscle fibers thereby leading to an increase in the proportion of fast fiber in the muscle composition. Such an inhibitor will serve to prevent the effect of calcineurin being mediated through an interaction between NFAT and MEF2 (or other transcription factors that bind to a dephosphorylated or activated NFAT protein). Thus, an inhibitor as described in this embodiment may be one which inhibits the dephosphorylation of NFAT, inhibits the expression of NFAT or one which prevents the interaction of NFAT and MEF2.

iii. Candidate Substances

As used herein the term "candidate substance" refers to any molecule that may potentially modulate calcineurin activity. The candidate substance may be a protein or fragment thereof, a small molecule, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to other known modulators of calcineurin activity. For example, known activators of calcineurin include Ca2+ and calmodulin whereas known inhibitors include cyclosporin A

and FK506. Such an endeavor often is know as "rational drug design," and includes not only comparisons with know inhibitors, but predictions relating to the structure of target molecules.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or target compounds. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for an NFAT molecule, or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches.

It also is possible to use antibodies to ascertain the structure of a target compound or inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotype would be expected to be an analog of the original antigen. The anti-idiotype could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

On the other hand, one may simply acquire, from various commercial sources, small molecule libraries that are believed to meet the basic criteria for useful drugs in an effort to "brute force" the identification of useful compounds. Screening of such libraries, including combinatorially generated libraries (*e.g.*, peptide libraries), is a rapid and efficient way to screen large number of related (and unrelated) compounds for activity. Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third and fourth generation compounds modeled of active, but otherwise undesirable compounds.

Candidate compounds may include fragments or parts of naturally-occurring compounds or may be found as active combinations of known compounds which are otherwise

inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be polypeptide, polynucleotide, small molecule or any other compounds that may be designed through rational drug design starting from known modulators of calcineurin.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly increase or decrease the calcineurin activity from the cell in comparison to their normal levels. Compounds that achieve significant appropriate changes in muscle cell phenotype will be used.

Significant changes in muscle cell phenotype, *e.g.*, as measured using myocyte growth, Ca^{2+} response, muscle fiber specific gene expression, and the like are represented by a changes in activity of at least about 30%-40%, and most preferably, by changes of at least about 50%, with higher values of course being possible. The active compounds of the present invention also may be used for the generation of antibodies which may then be used in analytical and preparatory techniques for detecting and quantifying further such modulators.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

b. *In vitro* Assays

A quick, inexpensive and easy assay to run is a binding assay. Binding of a molecule to a target may, in and of itself, be alter the activity of calcineurin, due to steric, allosteric or charge-charge interactions. This can be performed in solution or on a solid phase and can be utilized as a first round screen to rapidly eliminate certain compounds before moving into more sophisticated screening assays. In one embodiment of this kind, the screening of compounds that bind to the calcineurin molecule or fragment thereof is provided

The target may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the target or the compound may be labeled, thereby permitting determining of binding. In another embodiment, the assay may measure the inhibition of binding of a target to a natural or artificial substrate or binding partner (such as NFAT and MEF2). Competitive binding assays can be performed in which one of the agents (NFAT3 for example) is labeled. Usually, the target will be the labeled species, decreasing the chance that the labeling will interfere with the binding moiety's function. One may measure the amount of free label versus bound label to determine binding or inhibition of binding.

A technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with, for example, NFAT and washed. Bound polypeptide is detected by various methods.

Purified target, such as NFAT, can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the polypeptide can be used to immobilize the polypeptide to a solid phase. Also, fusion proteins containing a reactive region (preferably a terminal region) may be used to link an active region (*e.g.*, the C-terminus of NFAT) to a solid phase.

c. ***In cyto* Assays**

Various cell lines that exhibit characteristics of fast or slow muscle fiber type can be utilized for screening of candidate substances. Such cells can be used in assays in which the compound would be formulated appropriately, given its biochemical nature, and contacted with a target cell.

Depending on the assay, culture may be required. As discussed above, the cell may then be examined by virtue of a number of different physiologic assays (growth, size, Ca^{++} effects, fiber specific gene expression). Alternatively, molecular analysis may be performed in which the function of calcineurin and related pathways may be explored. This involves assays such as those for protein expression, enzyme function, substrate utilization, mRNA expression (including differential display of whole cell or polyA RNA) and others.

d. *In vivo* Assays

The present invention particularly contemplates the use of various animal models. Here, transgenic mice expressing a constitutively activated calcineurin can be used to monitor the effects of the candidate substance in a whole animal system. The generation of these animals has been described elsewhere in this document. These models can, therefore be used not only screen for modulators of muscle specific fiber.

Treatment of these animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route the could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are systemic intravenous injection, regional administration via blood or lymph supply.

Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Such criteria include, but are not limited to, survival, alteration in muscle size or mass, and improvement of general physical state including activity. It also is possible to perform histologic studies on tissues from these mice, or to examine the molecular state of the cells, which includes cell size or alteration in the expression of muscle fiber specific genes. U.S. Patent 5,628,328 provides methods for determining muscle mass in a human subject useful for monitoring athletic conditioning, weight loss programs, nutritional deficiencies, and disease states which cause muscle wasting.

6. **Pharmaceutical Compositions**

Where clinical application of an active ingredient (drugs, polypeptides, antibodies or liposomes containing antisense oligo- or polynucleotides or expression vectors) is undertaken, it will be necessary to prepare a pharmaceutical composition appropriate for the intended application. Generally, this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate buffers to render the complex stable and allow for uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the active ingredient, as discussed above, further dispersed in pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of therapeutic compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact

concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, a controlled release patch, salve or spray.

The therapeutic compositions of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. A preferred embodiment delivery route, for the treatment of a disseminated disease state is systemic, however, regional delivery is also contemplated.

An effective amount of the therapeutic composition is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection desired.

Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the patient, the route of administration, the intended goal of treatment and the potency, stability and toxicity of the particular therapeutic substance.

7. Transgenic Animals/Knockout Animals

In one embodiment of the invention, transgenic animals are produced which contain a functional transgene encoding a functional calcineurin polypeptide or variants thereof. Transgenic animals expressing calcineurin transgenes, recombinant cell lines derived from such animals and transgenic embryos may be useful in methods for screening for and identifying agents that induce or repress function of calcineurin. Alternative transgenic animals that may be employed herein include those which have a functional NFAT transgene. Transgenic animals of the present invention also can be used as models for studying indications such as muscular dystrophy.

In one embodiment of the invention, a calcineurin transgene is introduced into a non-human host to produce a transgenic animal expressing a human or murine calcineurin gene. The transgenic animal is produced by the integration of the transgene into the genome in a manner that permits the expression of the transgene. Methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Patent 4,873,191; which is incorporated herein by reference), Brinster *et al.*, 1985; which is incorporated herein by reference in its entirety) and in "Manipulating the Mouse Embryo; A Laboratory Manual" 2nd edition (eds., Hogan, Beddington, Costantimi and Long, Cold Spring Harbor Laboratory Press, 1994; which is incorporated herein by reference in its entirety).

It may be desirable to replace the endogenous calcineurin by homologous recombination between the transgene and the endogenous gene; or the endogenous gene may be eliminated by deletion as in the preparation of "knock-out" animals. Typically, a specific gene flanked by genomic sequences is transferred by microinjection into a fertilized egg. The microinjected eggs are implanted into a host female, and the progeny are screened for the expression of the transgene. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, mammals, and fish. Within a particularly preferred embodiment, transgenic mice are generated which overexpress calcineurin or express a mutant form of the polypeptide. Alternatively, the absence of a calcineurin in "knock-out" mice permits the study of the effects that loss of calcineurin protein has on a cell *in vivo*. Knock-out mice also provide a model for the development of calcineurin-related defects in muscle development.

As noted above, transgenic animals and cell lines derived from such animals may find use in certain testing experiments. In this regard, transgenic animals and cell lines capable of expressing wild-type or mutant calcineurin may be exposed to test substances. These test substances can be screened for the ability to enhance wild-type calcineurin expression and or function or impair the expression or function of mutant calcineurin.

8. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus, can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Materials and Methods

Cell culture and transfection conditions

NIH 3T3 cells or C2C12 myogenic cells were cultured, transfected with plasmid vectors, and assayed for luciferase and ß-galactosidase, as previously described (Grayson *et al.*, 1995; Grayson *et al.*, 1998). Each 35 mm dish of cells was cotransfected with promoter-reporter plasmid (0.5 μg), an expression plasmid that uses the CMV promoter to force expression of a constitutively active form of calcineurin (Manalan and Klee, 1983; O'Keefe *et al.*, 1992), or empty vector (pCI-NEO; 0.5 μg), along with a CMV-lacZ plasmid (0.5 μg) as an internal control for transfection efficiency. For dose-response experiments (FIG. 2), the total input DNA and the amount of promoter-reporter plasmid and CMV-lacZ was held constant, but the ratio of calcineurin expression vector to empty vector was varied.

Plasmid constructions

The expression plasmid used to stimulate calcineurin-regulated gene transcription was constructed by linking a CMV promoter carried in pCI-NEO (Promega) to a truncated variant of calcineurin A from which the carboxyl terminal region containing the autoinhibitory domain and a portion of the calmodulin binding domain was deleted (O'Keefe *et al.*, 1992). This form

of calcineurin exhibits constitutive phosphatase activity, and is not subject to regulation by calcium-calmodulin in the manner of the native protein (O'Keefe *et al.*, 1992). Promoter-reporter constructs were designed by linking the luciferase gene carried in pGL3 (Promega) to upstream promoter regions from the myoglobin (Mb), troponin I slow (TnIs), and muscle creatine kinase (MCK) genes, each of which have been shown previously to recapitulate the expression pattern of the respective endogenous genes when linked to a reporter gene and introduced into transgenic mice (Parsons *et al.*, 1993; Levitt *et al.*, 1995; Shield *et al.*, 1996).

Other promoter-reporter plasmids used as controls (CMV-luciferase; TATA-luciferase; CMV-lacZ), or to identify upstream regulatory elements involved in transducing the signal derived from activated calcineurin (Mb380; MbΔA/T; MbΔCCAC; CCAC-TATA; A/T-TATA; CCAC-A/T-TATA) have been described in previous publications from this laboratory (Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995; Grayson *et al.*, 1998). Reporter constructions bearing five copies of the upstream NFAT response element from the myoglobin promoter (NRE-TATA and NRE-CCAC-A/T-TATA) were based on the oligonucleotide sequence 5'-AACCAGGAAATAGGATGCCCT-3' (SEQ ID NO:1), and its complementary strand, representing nucleotide positions -694 to -674 in the human myoglobin promoter (underlined bases illustrate the NFAT consensus binding motif).

Putative NFAT binding sites within the myoglobin and troponin I slow promoters were disrupted using a PCR-based mutagenesis procedure, as described (Yang *et al.*, 1997). The specific nucleotide sequence modifications included: myoglobin promoter (-690) AGGAAATA to GTCGACTA and (-232, reverse strand) TGGAAAGA to CTCGAGGA; TnI slow promoter (-738) AGGAAAC to AGCTAGC and (-639) TGGAAACA to ACTAGTCA.

Plasmids used to express NFAT-GFP fusion proteins were constructed in pEGFP-N1 (Clontech), using cDNA sequences encoding full length (amino acids 1-716) or truncated (amino acids 319-716) NFATc (Northrop *et al.*, 1994), modified at the carboxyl terminus for fusion in the correct reading frame to GFP. In the construct designed to express the truncated NFATc-GFP fusion protein (ΔNFATc-GFP), the native leucine residue at position 319 was converted to a methionine initiation codon. In both NFATc-GFP and ΔNFATc-GFP, the native stop codon was replaced with a 7 amino acid insertion preceeding the GFP coding sequence.

Fluorescence microscopy

An Olympus IMT-2 inverted fluorescence photomicroscope with FITC illumination and detection was used for evaluation and photography of C2C12 cells transfected with GFP expression plasmids. GFP fluorescence (excitation peak = 488nm, emission peak = 507nm) was photographed with Kodak Elite II 400 ASA slide film using an Olympus SC35 SLR camera back.

Histochemical analysis of fiber type in muscles from intact animals

Adult rats were treated with cyclosporin A (5 mg/kg) or vehicle administered by intraperitoneal injection daily for 6 weeks. Animal care was in accordance with institutional guidelines. Sections of soleus muscles from 7 animals in each group were histochemically stained for myosin ATPase activity at pH 4.54, as described (Brooke and Kaiser, 1970). The proportion of fast and slow fibers was quantified by three observers who were blinded to the treatment status of the animals. Fibers expressing fast myosin were identified in 8μm cryosections sections of the same muscles, post-fixed in 4% paraformaldehyde, by immunohistochemical analysis using a commercially available mouse monoclonal antibody (MY-32: Sigma, St. Louis, MO; 1:400) and LRSC goat anti-mouse IgG (Jackson Immunochemicals, West Grove, PA; 1:50). Nuclei were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR) at 0.6μg/ml for 10 minutes.

EXAMPLE 2

Selective Activation of Slow Fiber-Specific Promoters By Forced Expression Of a Constitutively Active Form Of Calcineurin

The myoglobin and troponin I slow (TnIs) genes are expressed selectively in slow, oxidative skeletal muscle fibers (Levitt *et al.*, 1995; Garry *et al.*, 1996), while the muscle creatine kinase (MCK) gene is expressed most abundantly in the fast, glycolytic myofiber subtype (Yamashita and Yoshioka 1991). To test whether these genes might respond differently to a calcineurin-stimulated signaling pathway, skeletal myogenic cells were transfected with reporter genes linked to well-characterized control regions from these genes, along with an expression vector encoding a constitutively active (calcium-insensitive) form of calcineurin that retains sensitivity to inhibition by cyclosporin A (Manalan and Klee, 1983; O'Keefe *et al.*, 1992).

Transcriptional activity of the slow fiber-specific myoglobin and TnIs promoters was stimulated in cultured skeletal myotubes (C2C12) by active calcineurin, as measured by expression of luciferase in cotransfection assays (FIG. 1). In contrast, activity of the fast-fiber specific MCK promoter, or of other strong (CMV) or weak (minimal TATA element) promoters, was unaffected by activated calcineurin. The induction of the myoglobin promoter in the presence of the calcineurin expression plasmid was inhibited by cyclosporin A. This result indicates the specificity of the response, since the effect of cyclosporin A is to bind cyclophilin and form a protein complex that binds calcineurin and inhibits its protein phosphatase activity (Liu *et al.*, 1991). The same relative potency of calcineurin-dependent transactivation (myoglobin and TnIs >> MCK, CMV, or TATA) was observed in Sol8 myotubes, a different myogenic cell line. In contrast, forced expression of activated calcineurin had no effect on promoter activity in undifferentiated myoblasts or in 3T3 fibroblasts (FIG. 1), demonstrating a requirement for muscle-specific factors in the calcineurin-stimulated pathway for transcriptional control of the myoglobin and TnIs promoters.

EXAMPLE 3

Calcineurin-stimulated trans-activation of slow fiber-specific promoters requires nucleotide sequence motifs characteristic of NFAT binding sites

The finding that the myoglobin and TnIs promoters can be transcriptionally regulated by a calcineurin-dependent mechanism suggested the participation of NFAT transcription factors in the signaling cascade. Examination of the complete nucleotide sequences of these functionally defined transcriptional control regions (2.0 and 4.2 kb, respectively) revealed two 8 bp elements within each that match the consensus binding sequence for NFAT transcription factors (Rao *et al.*, 1997). The response to activated calcineurin of the native promoter sequences was compared to that of mutated promoters in which these putative NFAT recognition elements were ablated by site-directed mutatagenesis.

Disruption of putative NFAT recognition elements within both the myoglobin (FIG. 2A) and TnIs (FIG. 2B) promoters diminished the response to activated calcineurin, indicating that the transactivation mechanism is likely to involve DNA binding of NFAT proteins. Transduction of the calcineurin-directed signal to the native myoglobin and TnIs promoters exhibited a saturable dose-response relationship with respect to the activated calcineurin

expression plasmid, and diminished reporter gene activation was evident across the entire dose range examined. Some degree of calcineurin-dependent transactivation persisted after ablation of identifiable NFAT binding sites within these transcriptional control regions. Thus, either cryptic binding sites for NFAT proteins that cannot be recognized by inspection of the DNA sequence are present, or calcineurin-dependent signaling to these promoters can be driven without direct DNA binding of NFAT proteins.

Nuclear localization of NFAT proteins in skeletal myocytes is under the control of calcineurin (FIG. 2C), as predicted from previously published results in lymphocytes (Timmerman *et al.*, 1996). A fusion protein linking Green Fluorescent Protein (GFP) to full length NFATc (NFATc-GFP) is excluded from the nucleus in C2C12 cells under basal conditions, but undergoes nuclear translocation in the presence of activated calcineurin. As controls, an NFATc-GFP fusion protein lacking amino acids 1-318 of NFATc (ΔNFATc-GFP) is constitutively localized to the nucleus in the absence of activated calcineurin, while native GFP is distributed across both cytoplasmic and nuclear compartments. The amino terminal segment of NFAT proteins (missing in ΔNFATc-GFP) includes the conserved SPRIEIT motif that constitutes the calcineurin targeting site (Arambaru *et al.*, 1998).

EXAMPLE 4

Calcineurin-stimulated trans-activation of slow fiber-specific promoters requires collaboration among multiple transcription factors

Muscle-specific transcription factors are required for calcineurin-dependent activation of the myoglobin and TnI slow promoters, since no response was observed in a fibroblast cell background (FIG. 1). Previously, two conserved upstream response elements have been defined within the myoglobin promoter, both of which are required for transcriptional activity in skeletal myotubes or cardiac myocytes (Devlin *et al.*, 1989; Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995; Grayson *et al.*, 1998). These CCAC and A/T elements represent binding sites for Sp1 and MEF2 proteins, respectively, and function synergistically in muscle-specific gene regulation (Grayson *et al.*, 1995; Grayson *et al.*, 1998). This prior work established a molecular basis for muscle-specific expression of myoglobin, but failed to account for selective expression of myoglobin in slow fiber types, since Sp1 and MEF2 proteins are equally abundant in slow and fast fibers.

A myoglobin promoter segment truncated to nucleotides -373 to +7 (Mb380) was sufficient for muscle-specific expression in prior experiments (Devlin *et al.*, 1989; Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995) and was responsive to calcineurin stimulation in the current studies (FIG. 3A). This region includes the CCAC and A/T motifs required for muscle-specific promoter activity, as well as a putative NFAT response element. Nucleotide substitutions within either the CCAC or A/T elements of Mb380 reduced basal transcription in differentiated myotubes, as observed previously, and abrogated the response to calcineurin (FIG. 3A). Thus, mutations that compromise binding of MEF2, Sp1, or other factors to the CCAC and A/T elements interdict the calcineurin-stimulated response, even when the NFAT consensus binding motif at -232 remains intact.

Functional interactions between transcription factors binding to motifs within the myoglobin promoter were examined further by linkage of various combinations of multimerized oligonucleotide cassettes representing cognate binding sites for MEF2, Sp1 and NFAT in promoter-reporter constructions. As assessed by cotransfection assays in C2C12 myotubes (FIG. 3B), forced expression of activated calcineurin only marginally enhanced transcription (< 2-fold) of a construct bearing multiple copies of the CCAC motif. The response to calcineurin was somewhat more robust (3-fold) if multimers of the A/T element were included within the synthetic promoters, either in the absence of heterologous protein binding sites, or when combined with multimerized CCAC sites. A reporter construction bearing multiple copies of the upstream (-690) NFAT response element (NRE) from the myoglobin promoter was minimally stimulated by activated calcineurin (< 2-fold) in this cell background, but a construct combining NRE, A/T and CCAC motifs was potently transactivated (6-fold). These results demonstrate that collaborative interactions among proteins binding to NRE, A/T and CCAC elements from the myoglobin promoter are necessary for optimal transduction of the calcineurin-stimulated signal.

EXAMPLE 5

Administration of the calcineurin antagonist cyclosporin A to intact animals promotes slow-to-fast fiber transformation

To determine whether calcineurin-dependent activation of slow fiber-specific promoters observed in cultured myotubes is pertinent to mature myofibers of intact animals, the proportion of fast versus slow fibers was assessed within soleus muscles of rats treated with

cyclosporin A, a specific inhibitor of calcineurin (Liu *et al.*, 1991; Clipstone and Crabtree 1992). The intraperitoneal administration of an immunosuppressant dose (5 mg/kg/day) of cyclosporin A for 6 weeks uniformly increased the proportion of fast fibers defined either by histochemical staining of myosin ATPase activity (Brooke and Kaiser, 1970), or by specific immunohistochemical staining of fast skeletal myosin. In soleus muscles of 7 control animals, fast (Type II) fibers represented 4-24% (mean 14 $\pm$ 3%) of the total cell population, while 28-37% (mean 31 $\pm$ 1%) of soleus fibers expressed fast myosin in 7 cyclosporin A-treated rats ($p<.001$) (FIG. 4). This result is consistent with the hypothesis that physiological signals acting to establish and maintain the slow, oxidative myofiber phenotype in intact animals are transduced by a calcineurin-dependent pathway. Interdiction of the calcineurin-signaling pathway with cyclosporin A has reciprocal effects on expression of fast and slow myosin isoforms: not only is slow myosin expression reduced but fast myosin expression is enhanced. Thus, calcineurin-dependent signaling both activates slow fiber-specific genes and represses the fast fiber-specific program.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Aramburu *et al.*, *Molec. Cell.*, 1:627-637, 1998.

Arya *et al.*, *Hum Gene Ther.*, 9(9):1371-1380, 1998.

Baichwal and Sugden, In: *Gene Transfer*, Kucherlapati R, ed., New York, Plenum Press, 117-148, 1986.

Bassel-Duby, *et al.*, *Circ. Res.*, 73:360-366, 1993.

Batterson and Roizman, *J. Virol.*, 46:371-377, 1983.

Bedzyk *et al.*, *J. Biol. Chem.*, 265:18615, 1990.

Bellon *et al.*, *de Ses Filiales*, 190(1):109-142, 1996.

Benvenisty and Neshif, *Proc. Nat'l. Acad. Sci. USA*, 83:9551-9555, 1986.

Berns and Bohenzky, *Adv. Virus Res.*, 32:243-307, 1987.

Berns and Giraud, *Curr. Top. Microbiol. Immunol.*, 218:1-23, 1996.

Berns, *Microbiol Rev.*, 54:316-329, 1990.

Bertran *et al.*, *J. Virol.*, 70(10):6759-6766, 1996.

Black, B.L. and E.N. Olson, *Ann. Rev. Cell Dev. Biol.*, 14:167-196, 1998.

Blomer *et al.*, *J. Virol.*, 71(9):6641-6649, 1997.

Booth and Baldwin. In *The Handbook of Physiology: Integration of Motor, Circulatory, Respiratory and Metabolic Control During Exercise* (ed. L.B. Rowell and J.T.Shepard), pp.1075-1123. Bethesda:American Physiology Society, 1996.

Borensztajn *et al.*, *Am. J. Physiol.*, 229(2):394-397, 1975.

Briggs and Schachat, *Am. J. Physiol.*, 270(1 Pt 1):C298-C305, 1996.

Brinster *et al.*, *Proc. Nat'l Acad. Sci. USA*, 82:4438-4442, 1985.

Brooke and Kaiser, *Arch. Neurol.*, 23:369-379, 1970.

Caiozzo, *et al.*, *J. Appl. Physiol.*, 76:1764-1773, 1994.

Cameron *et al.*, *Cell*, 83:463-472, 1995.

Chaudhary *et al.*, *Proc. Nat'l. Acad. Sci.*, 87:9491, 1990.

Chen and Okayama, *Mol. Cell Biol.*, 7:2745-2752, 1987.

Chin and Allen, *Physiologist*, 39:A-75, 1996.

Clipstone and Crabtree, *Nature*, 357:695-697, 1992.

Coffin, *In:* Fields BN, Knipe DM, ed., VIROLOGY. New York: Raven Press, pp. 1437-1500, 1990.

Couch *et al., Am. Rev. Resp. Dis.*, 88:394-403, 1963.

Delp *et al., J. Appl. Physiol.*, 83(4):1291-1299, 1997.

DeLuca *et al., J. Virol.*, 56:558-570, 1985.

Devlin *et al., J. Biol. Chem.*, 264:13896-13900, 1989.

DiMario *et al., Nature*, 362:165-167, 1993.

Dolmetsch *et al., Nature*, 386:855-858, 1997.

Dubensky *et al., Proc. Nat'l. Acad. Sci. USA*, 81:7529-7533, 1984.

Elroy-Stein *et al., Proc. Nat'l Acad. Sci. USA*, 1989.

Elshami *et al., Gene Therapy*, 7(2):141-148, 1996.

Emmel *et al., Science*, 246:1617-1620, 1989.

Fechheimer *et al., Proc. Nat'l. Acad. Sci. USA*, 84:8463-8467, 1987.

Ferkol *et al., FASEB J.*, 7:1081-1091, 1993.

Fraley *et al., Proc. Nat'l. Acad. Sci. USA*, 76:3348-3352, 1979.

French *et al., Circulation*, 90(5):2414-2424, 1994.

Freshner, In *Animal Cell Culture: a Practical Approach* Second Edition, Oxford/New York, IRL Press, Oxford University Press, 1992.

Garry *et al., Dev. Genet.*, 19: 146-156, 1996.

Ghosh-Choudhury *et al., EMBO J.*, 6:1733-1739, 1987.

Ghosh and Bachhawat, (Wu G, Wu C ed.), New York: Marcel Dekker, pp. 87-104, 1991.

Ginsberg *et al., Proc. Nat'l Acad. of Sci. USA*, 88(5)1651-1655, 1991.

Glorioso *et al., Ann. Rev. Microbiol.*, 49:675-710, 1995.

Gomez-Foix *et al., J. Biol. Chem.*, 267:25129-25134, 1992.

Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.

Graham and Prevec, *Biotechnol.*, 20:363-390, 1992.

Graham and Prevec, *In:* E.J. Murray (ed.), Methods in Molecular Biology: Gene Transfer and Expression Protocol, Clifton, NJ: Humana Press, 7:109-128, 1991.

Graham and Van Der Eb, *Virology*, 52:456-467, 1973.

Graham *et al., J. Gen. Virol.*, 36:59-72, 1977.

Grayson *et al., Mol. Cell. Biol.*, 15: 1870-1878, 1995.

Grayson *et al... J. Cell. Biochem.*, 1998.

Grunhaus and Horwitz, *Seminar in Virology*, 3:237-252, 1992.

Harland and Weintraub, *J. Cell Biol.*, 101:1094-1099, 1985.

Hennig, and Lomo, *Nature*, 314: 164-166, 1985.

Hersdorffer *et al.*, *DNA Cell Biol.*, 9:713-723, 1990.

Herz and Gerard, *Proc. Nat'l. Acad. Sci. USA*, 90:2812-2816, 1993.

Hocy *et al.*, *Immunity*, 2: 461-472, 1995.

Hogan *et al.*, "Manipulating the Mouse Embryo A Laboratory Manual" 2nd edition eds., Hogan, Beddington, Costantimi and Long, Cold Spring Harbor Laboratory Press, 1994.

Hoh, *Curr. Opin. Rheumatol.*, 4(6):801-808,1992.

Holland *et al.*, *Virology*, 101:10-18, 1980.

Holloszy and Coyle, *J. Appl. Physiol.*, 56: 831-838, 1984.

Honess and Roizman, *J. Virol.*, 14:8-19, 1974.

Honess and Roizman, *J. Virol.*, 16:1308-1326, 1975.

Howlett and Willis WT, *Biochim. Biophys. Acta*, 1363(3):224-230, 1998

Hughes *et al.*, *Mech. Dev.*, 61: 151-163, 1997

Ianuzzo *et al.*, *J. Appl. Physiol.*, 70: 2328-2830.

Ivy JL, *Sports Med.*, 24(5):321-336, 1997

Izumo, S. and V. Mahdavi, *Nature*, 334: 539-542, 1988.

Johnson *et al.*, *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, eds., Chapman and Hall, New York, 1993.

Jones and Shenk, *Cell*, 13:181-188, 1978.

Jostarndt *et al.*, *J. Histochem. Cytochem.*, 44(10):1141-1152, 1996

Kaciuba-Uscilko *et al.*, *Am J Physiol*, 238(6):E518-E523, 1980

Kaneda *et al.*, *Science*, 243:375-378, 1989.

Karlsson *et al.*, *EMBO J.*, 5:2377-2385, 1986.

Kato *et al.*, *J. Biol. Chem.*, 266:3361-3364, 1991.

Kearns *et al.*, *Gene Ther.*, 3:748-755, 1996.

Klein *et al.*, *Nature*, 327:70-73, 1987.

Kong *et al.*, *J. Biol Chem.*, 269(17): 12963-12967, 1994.

Kong *et al.*, *J. Biol. Chem.*, 269: 12963-12967, 1994.

Kotin and Berns, *Virol.*, 170:460-467, 1989.

Kotin *et al.*, *Genomics*, 10:831-834, 1991.

Kotin *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 87:2211-2215, 1990.

Kudoh *et al., Circ. Res.*, 80:139-146, 1997.

Lam *et al.,. J. Biol. Chem.*, 270: 26511-26522, 1995.

Le Gal La Salle *et al., Science*, 259:988-990, 1993.

Levitt *et al., DNA Cell Biol.*, 14: 599-607, 1995.

Levitt *et al., DNA Cell Biol.* 14(7):599-607, 1995.

Levrero *et al., Gene*, 101:195-202, 1991.

Li *et al.,. Cell*, 71: 1181-1194, 1992.

Liu *et al., Cell*, 66: 807-815, 1991.

Mackie *et al., J. Appl. Physiol.*, 49(5):851-855, 1980.

Mair *et al., J. Appl. Physiol.*, 72(2):656-663, 1992.

Manalan, A.S. and C.B. Klee, *Proc. Nat'l. Acad. Sci., U S A* 80: 4291-4295, 1983.

Mann *et al., Cell*, 33:153-159, 1983.

Marette *et al., Am. J. Physiol.*, 263(2 Pt 1):C443-C452, 1992.

Markowitz *et al., J. Virol.*, 62:1120-1124, 1988.

Massie *et al., Circulation*, 78: 320-326, 1988.

Matsumoto *et al., J. Biotech. Histochem.*, 72(4):191-197, 1997.

McCaffery *et al., Science*, 262:750-754, 1993.

Mercier *et al., Amer. J. Resp. Crit. Care Med.*, 151: 1532-1536, 1995.

Michel *et al., J. Clin. Invest.*, 94: 277-285, 1994.

Mizukami *et al., Virology*, 217:124-130, 1996.

Molkentin *et al., Cell*, 93: 1-20, 1998.

Molkentin, J.D., and E.N. Olson, *Proc. Nat'l. Acad. Sci. USA*, 93, 9366-9373, 1996

Mulligan, *Science*, 260:926-932, 1993.

Myers, EPO 0273085

Nakano *et al., Brain Res. Bull.*, 43(2):149-154, 1997.

Nakayama *et al., Mol. Cell. Biol.*, 16(5):2408-2417, 1996.

Nakayama *et al., Mol. Cell. Biol.*, 16: 2408-2417 1996.

Neufer *et al., Am. J. Physiol.*, 271: C1828-1837, 1996.

Nicolas and Rubenstein, *In: Vectors: A survey of molecular cloning vectors and their uses.* Rodriguez and Denhardt (eds.), Stoneham: Butterworth, pp. 494-513, 1988.

Nicolau and Sene, *Biochem. Biophys. Acta*, 721:185-190, 1982.

Nicolau *et al., Methods Enzymol.*, 149:157-176, 1987.

Nishida *et al., J. Exp. Zool.*, 1;277(4):283-292, 1997.

Nolan, *Cell*, 77, 1-20, 1994.

Northrop *et al.*, *Nature*, 369: 497-502, 1994.

O'Keefe *et al.*, *Nature*, 357: 692-694, 1992.

Ogata and Yamasaki, *Anat. Rec.*, 248(2):214-223, 1997.

Ogawa *et al.*, *J. Mol. Med.*, 73:457-463, 1995.

Olson *et al.*, *Dev. Biol.*, 172: 2-14.

Ontell *et al.*, *Dev. Dynm.*, 198: 203-213, 1993.

Ostrove *et al.*, *Virology*, 113:532-533, 1981.

Parsons *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 90:1726-1730, 1993.

Paskind *et al.*, *Virology*, 67:242-248, 1975.

Perales *et al.*, *Proc. Nat'l. Acad. Sci.*, 91:4086-4090, 1994.

Pette and Vrbova, *Rev. Physiol. Biochem. Pharmacol*, 120:115-202, 1992.

Ponnazhagan *et al.*, *Hum. Gene Ther.*, 8:275-284, 1997a.

Ponnazhagan *et al.*, *J. Gen. Virol.*, 77:1111-1122, 1996.

Post *et al.*, *Cell*, 24:555-565, 1981.

Potter *et al.*, *Proc. Nat'l Acad. Sci. USA*, 81:7161-7165, 1984.

Qin *et al.*, *J. Biol. Chem.*, 272: 25210-25216, 1997.

Racher *et al.*, *Biotechnology Techniques*, 9:169-174, 1995.

Radler *et al.*, *Science*, 275:810-814, 1997.

Ragot *et al.*, *Nature*, 361:647-650, 1993.

Rao *et al.*, *Annu. Rev. Immunol.*, 15:707-747, 1997.

Rao *et al.*, *Mol. Cell. Biol.*, 16(7):3909-3922, 1996.

Renan, *Radiother. Oncol.*, 19:197-218, 1990.

Rich *et al.*, *Hum. Gene Ther.*, 4:461-476, 1993.

Ridgeway, *In:* Rodriguez RL, Denhardt DT, ed. Vectors: A survey of molecular cloning vectors and their uses. Stoneham: Butterworth, pp. 467-492, 1988.

Rippe *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.

Roizman and Sears, In *Fields' Virology*, 3rd Edition, eds. Fields *et al.*, (Raven Press, New York, N.Y.), pp. 2231-2295, 1995.

Rooney *et al.*, *EMBO J.*, 13:625-633, 1994.

Rosenfeld *et al.*, *Cell*, 68:143-155, 1992.

Rosenfeld *et al.*, *Science*, 252:431-434, 1991.

Roux *et al.*, *Proc. Nat'l Acad. Sci. USA*, 86:9079-9083, 1989.

Sabbah *et al.*, *Circulation*, 87:1729-1737, 1993.

Sadoshima and Izumo, *Circ. Res.*, 73:424-438, 1993b.

Sadoshima *et al.*, *Cell*, 75:977-984, 1993a.

Saltin and Gollnick In *Handbook of Physiology: Skeletal Muscle* (ed. L.D.D. Peachey), pp. 555-632. American Physiological Society, Bethesda. 1983.

Samulski *et al.*, *EMBO J.*, 10:3941-3950, 1991.

Schiaffino, S. and C. Reggiani, *Physiol. Rev.* 76:371-423, 1996.

Shen *et al.*, *Magn. Reson, Med.*, 36(1):30-38, 1996.

Shield *et al.*, *Mol. Cell. Biol.*, 16:5058-5068, 1996.

Shiraishi *et al.*, *Transplant Internationl*, 1-0(3):202-206, 1997.

Smith and Moss, *Gene*, 25:21-28, 1983.

Song *et al.*, *Science*, 275:536-540, 1997.

Sreter *et al.*, *Am. J. Physiol.*, 253:C296-C300, 1987.

Srivastava *et al.*, *J. Virol.*, 45:555-564, 1983.

Stemmer and Klee, *Biochemistry*, 33:6859-6866, 1994.

Stockdale, *Cell Struct. Funct.*, 22:37-43, 1997.

Stratford-Perricaudet and Perricaudet, *In: Human Gene Transfer*, Eds, O. Cohen-Haguenauer and M. Boiron, Editions John Libbey Eurotext, France, pp. 51-61, 1991.

Stratford-Perricaudet *et al.*, *Hum. Gene Ther.*, 1:241-256, 1990.

Su *et al.*, *Eur. J. Biochem.*, 230:469-474, 1995.

Sullivan *et al.*, *Circulation*, 79:324-329, 1989.

Temin, *In: Gene Transfer*, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986.

Tikkanen *et al.*, *Atherosclerosis*, 120:1-5, 1996.

Tikkanen *et al.*, *Atherosclerosis*, 120(1-2):1-5, 1996.

Timmerman *et al.*, *Nature*, 383:837-840, 1996.

Top *et al.*, *J. Infect. Dis.*, 124:155-160, 1971.

Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.

U.S. Patent 4,873,191

U.S. Patent 5,252,479

U.S. Patent 5,359,046

U.S. Patent 5,672,344

U.S. Patent 5,708,158

Varmus *et al.*, *Cell*, 25:23-36, 1981.

Vrbova, *J. Physiol. (Lond)*, 169:513-526, 1963.

Wagner *et al.*, *Proc. Nat'l. Acad. Sci.*, 87(9):3410-3414, 1990.

Walsh *et al.*,. *Biochemistry*, 34:5561-5568, 1995.

Webster *et al.*, *Cell*, 52:503-513, 1988.

Werthman *et al.*, *Journal of Urology*, 155(2):753-756, 1996.

Westerblad and Allen, *J. Gen. Physiol.*, 98:615-635, 1991.

Williams and Neufer, In *The Handbook of Physiology: Integration of Motor, Circulatory, Respiratory and Metabolic Control During Exercise* (ed. L.B. Rowell and J.T.Shepard), pp.1124-1150. Bethesda:American Physiology Society, 1996.

Williams *et al.*, *J. Comp. Physiol. [B]*, 167(8):527-535, 1997.

Williams *et al.*, *J. Biol. Chem.*, 261:376-380 1986.

Wong *et al.*. *Gene*, 10:87-94, 1980.

Wu & Wu, *Biochemistry*, 27:887-892, 1988.

Wu & Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.

Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.

Yamashita and Yoshioka, *J Muscle Res. Cell Motil.* 12: 37-44, 1991.

Yamazaki *et al.*, *J. Biol. Chem.*, 271:3221-3228, 1996.

Yang *et al.*, *Mol. Cell. Biol.*, 17:5236-5243 1997.

Yang *et al.*, *Proc. Nat'l. Acad. Sci USA*, 87:9568-9572, 1990.

Zou *et al.*, *J. Biol. Chem.*, 271:33592-33597, 1996.

CLAIMS:

1. A method of altering the phenotype of skeletal muscle tissue, comprising contacting said tissue with a modulator of calcineurin activity.

2. The method of claim 1, wherein the altering the phenotype comprises an increase of the proportion fast fiber to slow fiber in said tissue.

3. The method of claim 1, wherein the altering the phenotype comprises an increase of the proportion slow fiber to fast fiber in said tissue.

4. The method of claim 1, wherein said phenotype comprises a change in the size of said cells of said tissue.

5. The method of claim 1, wherein the muscle cells are derived from soleus, gastroenemius, quadriceps, tibialis anterior, pectordis, lattissimus dorsi, diaphragm, biceps, triceps, gluteus and tongue.

6. The method of claim 5, wherein the muscle cells are soleus muscle cells.

7. The method of claim 1, wherein the animal is human, murine, bovine, equine, porcine, ovine, canine, feline, rodent, avian or fish.

8. The method of claim 7, wherein the animal is a human.

9. The method of claim 1, wherein said altering the phenotype comprises an increase in expression of fiber-type specific gene expression.

10. The method of claim 9, wherein said fiber-type specific gene expression is specific to fast fiber cells.

11. The method of claim 10, wherein said gene expression comprises the expression of muscle creatinine kinase, fast myosin heavy chain, fast myosin light chain, fast troponin or parvalbumin.

12. The method of claim 9, wherein said fiber-type specific gene expression is specific to slow fiber cells.

13. The method of claim 12, wherein said gene expression comprises the expression of myoglobin, troponin I, slow myosin heavy chain, slow myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase.

14. The method of claim 1, wherein said modulator is an inhibitor of calcineurin activity.

15. The method of claim 13, wherein the inhibitor of calcineurin reduces the expression of calcineurin.

16. The method of claim 13, wherein the inhibitor of calcineurin is an agent that binds to and inactivates calcineurin.

17. The method of claim 13, wherein the inhibitor of calcineurin inhibits the interaction of calcineurin with an NFAT.

18. The method of claim 15, wherein the agent that reduces the expression of calcineurin is an antisense construct.

19. The method of claim 18, wherein the agent that binds to and inactivates calcineurin is an antibody or a small molecule inhibitor.

20. The method of claim 19, wherein the antibody is a single chain antibody.

21. The method of claim 19, wherein said antibody is a monoclonal antibody.

22. The method of claim 13, wherein said inhibitor is selected from the group consisting of cyclosporin, FK506, AP1510 and FK1012.

23. The method of claim 1, wherein said modulator is a stimulator of calcineurin activity.

24. The method of claim 23, wherein said stimulator of calcineurin activity increases the expression of calcineurin.

24. The method of claim 23, wherein said stimulator of calcineurin activity activates said calcineurin.

25. The method of claim 24, wherein said activator of calcineurin activity is calcium or calmodulin.

26. The method of claim 1, wherein said modulator of calcineurin activity increases the activity of an NFAT.

27. The method of claim 26, wherein said increase in an NFAT activity comprises stimulation of the dephosphorylation of an NFAT.

28. The method of claim 26, wherein said increase in an NFAT activity comprises increasing the expression of an NFAT.

29. The method of claim 26, wherein said increase in an NFAT activity comprises contacting NFAT with an agent that activates said NFAT.

30. The method of claim 26, wherein said increase in an NFAT activity comprises increasing the interaction of NFAT with MEF2.

31. The method of claim 26, wherein said NFAT is selected from the group consisting of NFAT1, NFAT2, NFAT3 and NFAT4.

32. The method of claim 1, wherein said modulator of calcineurin activity inhibits the activity of an NFAT.

33. The method of claim 32, wherein inhibition of the activity of an NFAT comprises inhibiting the dephosphorylation of NFAT.

34. The method of claim 32, wherein inhibition of the activity of an NFAT comprises reducing the expression of NFAT.

35. The method of claim 32, wherein inhibition of the activity of an NFAT comprises contacting NFAT with an agent that binds to and inactivates NFAT.

36. The method of claim 32, wherein inhibition of the activity of an NFAT comprises inhibiting the interaction of NFAT with MEF2.

37. The method of claim 34, wherein the agent that reduces the expression of an NFAT is an antisense construct.

38. The method of claim 35, wherein the agent that binds to and inactivates an NFAT is an antibody or a small molecule inhibitor.

39. The method of claim 38, wherein the antibody is a single chain antibody.

40. The method of claim 38, wherein said antibody is a monoclonal antibody.

41. The method of claim 32, wherein said NFAT is selected from the group consisting of NFAT1, NFAT2, NFAT3 and NFAT4.

42. A method of transforming a fast muscle fiber to a slow muscle fiber comprising increasing the calcineurin activity in said fast muscle fiber.

43. The method of claim 42, wherein said calcineurin is encapsulated in a liposome.

44. The method of claim 42, comprising the steps of:

(i) providing an expression construct comprising a first nucleic acid encoding an active calcineurin and promoter functional in said muscle fibers wherein said nucleic acid is under transcriptional control of said first promoter; and

(ii) contacting said expression construct with said fast muscle fiber in an amount effective to promote the activation of NFAT of said fiber;

wherein activation of NFAT in said fast fiber promotes the transformation of fast fiber to slow fiber.

45. The method of claim 42, wherein said fast fiber is located within an animal.

46. The method of claim 44, wherein said first nucleic acid is a cDNA or genomic DNA.

47. The method of claim 44, wherein said first expression construct is selected from the group consisting of an adenovirus, an adeno-associated virus, a vaccinia virus a herpes virus and lentivirus.

48. The method of claim 44, wherein said promoter is selected from the group consisting of CMV IE, SV40 IE, RSV, β-actin, tetracycline regulatable and ecdysone regulatable.

49. The method of claim 44, wherein said contacting is effected by direct injection of a muscle containing said slow fiber with said expression construct.

50. The method of claim 44, wherein said contacting comprises delivering said expression construct intravenously, subcutaneously, intramuscularly, or intraperitoneally to a muscle containing said fast fiber.

51. A method of transforming a slow muscle fiber to a fast muscle fiber comprising inhibiting calcineurin activity in said slow muscle fiber.

52. The method of claim 51, comprising the steps of:

(i) providing an expression construct comprising a first nucleic acid encoding a calcineurin gene positioned antisense to a promoter functional in said slow muscle fiber, wherein said nucleic acid is under transcriptional control of said first promoter; and

(ii) contacting said expression construct with said slow muscle fiber in an amount effective to decrease the calcineurin activity in said fiber;

wherein said decrease in calcineurin activity in said slow fiber promotes the transformation of slow fiber to fast fiber.

53. The method of claim 52, comprising contacting said slow muscle fiber with cyclosporin, FK506, AP1510 and FK1012.

54. The method of claim 52, comprising inhibiting the interaction of NFAT with MEF2.

55. A method of screening for modulators of muscle fiber phenotype comprising the steps of:

(a) providing a skeletal muscle cell expressing an NFAT and/or a MEF2 gene;

(b) contacting said cell with a candidate modulator; and

(c) monitoring said cell for a phenotype that is absent when said cell is not treated with said candidate modulator.

56. The method of claim 55, wherein said cell is in an animal.

57. The method of claim 55, wherein said cell is derived from a fast muscle cell line.

58. The method of claim 55, wherein said cell is derived from a slow muscle cell line.

59. The method of claim 55, wherein contacting is performed *in vitro*.

60. The method of claim 57, wherein said monitoring comprises measuring the activity or expression of a fast fiber-specific gene.

61. The method of claim 58, wherein said monitoring comprises measuring the activity or expression of a slow fiber-specific gene.

62. The method of claim 55, wherein said monitoring comprises measuring the size or mass of said cell.

63. The method of claim 55, wherein said monitoring comprises monitoring Ca^{++} response in said cell.

64. The method of claim 63, wherein monitoring said Ca^{++} response comprises monitoring Ca^{++} dependent gene expression in said cell.

65. The method of claim 55, wherein said contacting is performed *in vivo*.

66. The method of claim 55, wherein said candidate modulator is an antisense construct.

67. The method of claim 55, wherein said candidate modulator is from a small molecule library.

68. The method of claim 55, wherein said candidate modulator is an antibody.

69. The method of claim 68, wherein said antibody is a single chain antibody.

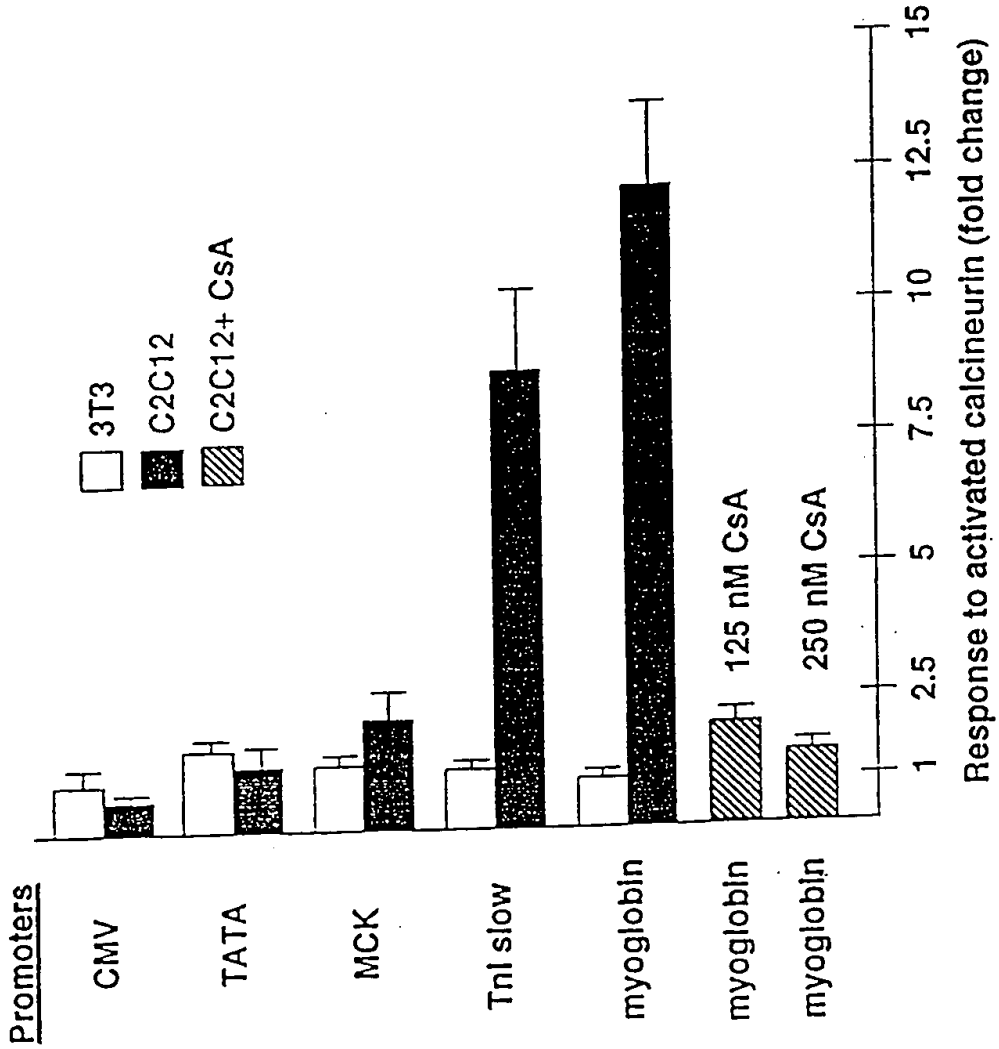

Promoters
CMV
TATA
MCK
Tnl slow
myoglobin
myoglobin
myoglobin
250 nM CsA
125 nM CsA
3T3
C2C12
C2C12+ CsA
1
2.5
5
7.5
10
12.5
15
Response to activated calcineurin (fold change)

FIG. 1

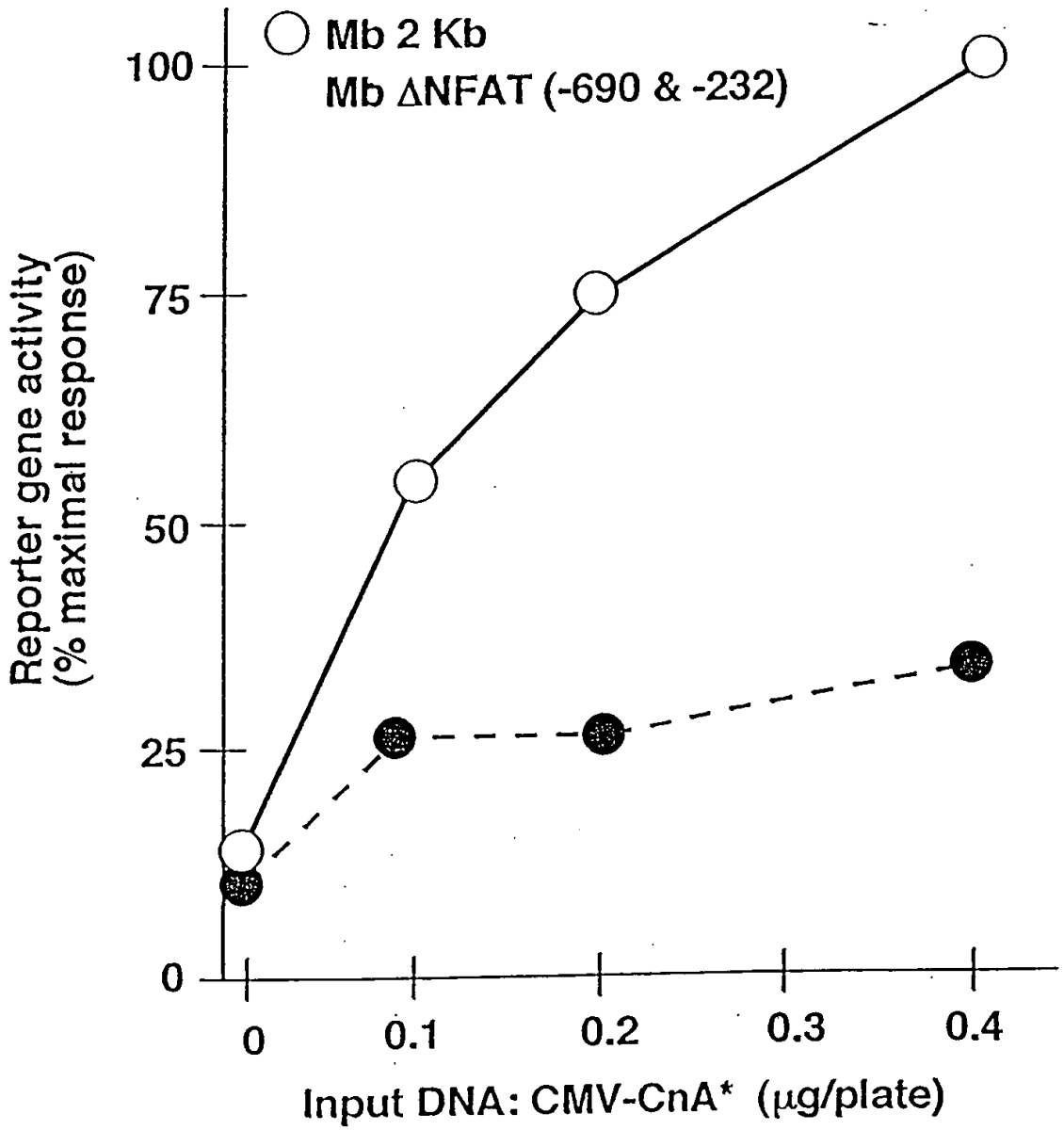

Mb 2 Kb
Mb ΔNFAT (-690 & -232)
Reporter gene activity
(% maximal response)
100
75
50
25
0
0
0.1
0.2
0.3
0.4
Input DNA: CMV-CnA* (μg/plate)

FIG. 2A

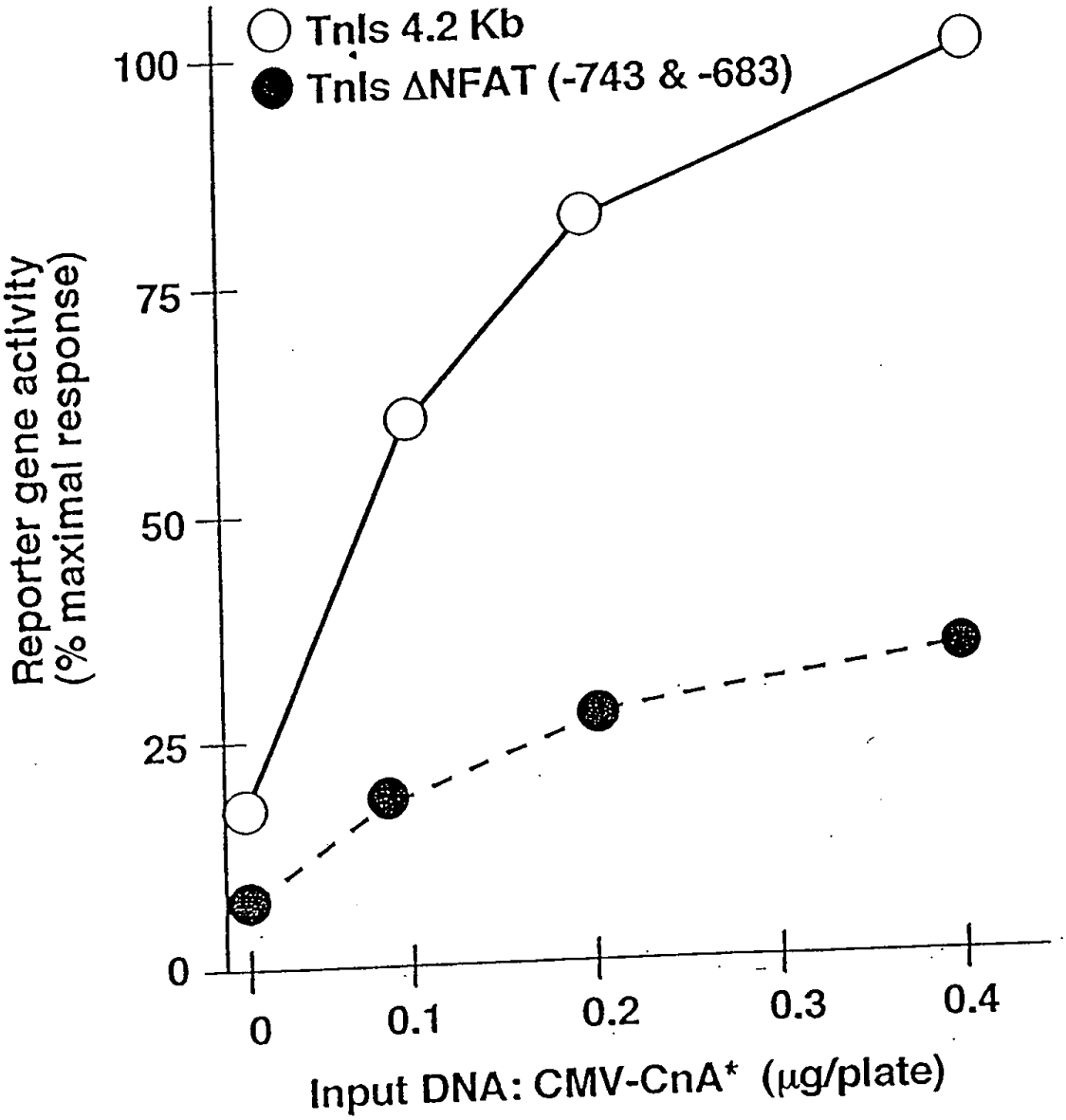

TnIs 4.2 Kb
TnIs ΔNFAT (-743 & -683)
Reporter gene activity
(% maximal response)
100
75
50
25
0
0
0.1
0.2
0.3
0.4
Input DNA: CMV-CnA* (μg/plate)

FIG. 2B

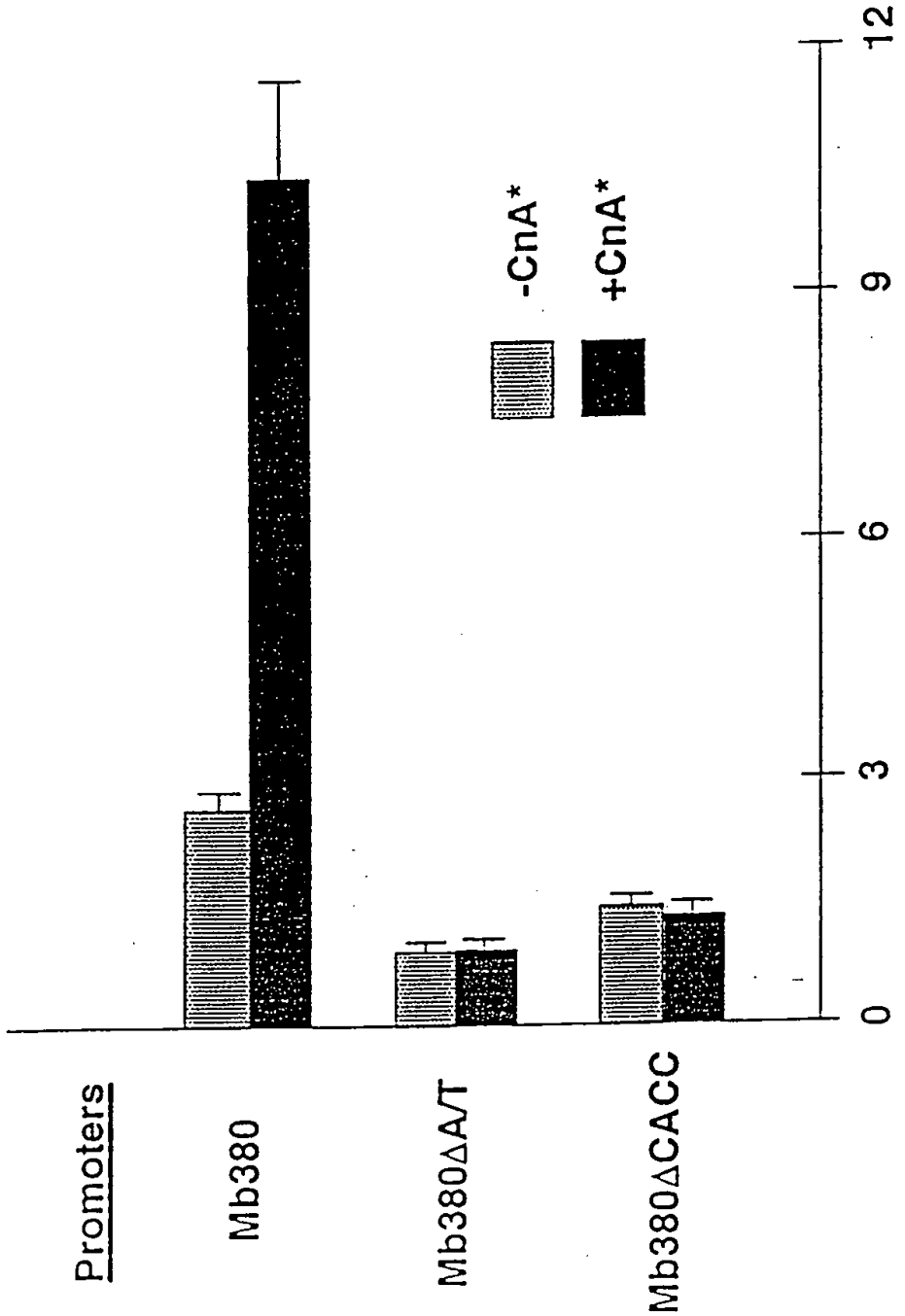

Promoters
Mb380
Mb380ΔA/T
Mb380ΔCACC
-CnA*
+CnA*
0
3
6
9
12
Luciferase activity

FIG. 3A

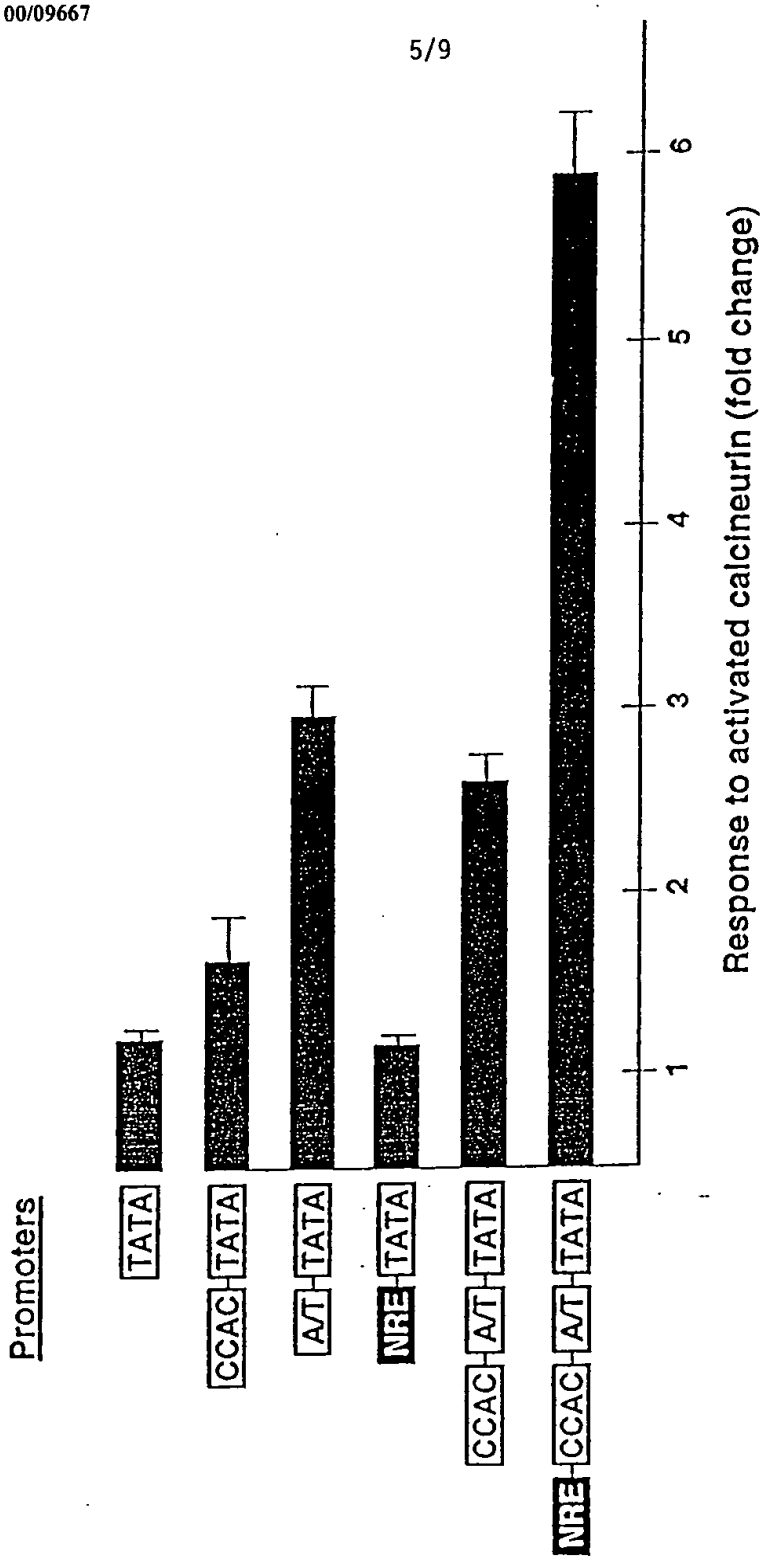

Promoters
TATA
CCAC TATA
A/T TATA
NRE TATA
CCAC A/T TATA
NRE CCAC A/T TATA
Response to activated calcineurin (fold change)
1
2
3
4
5
6

FIG. 3B

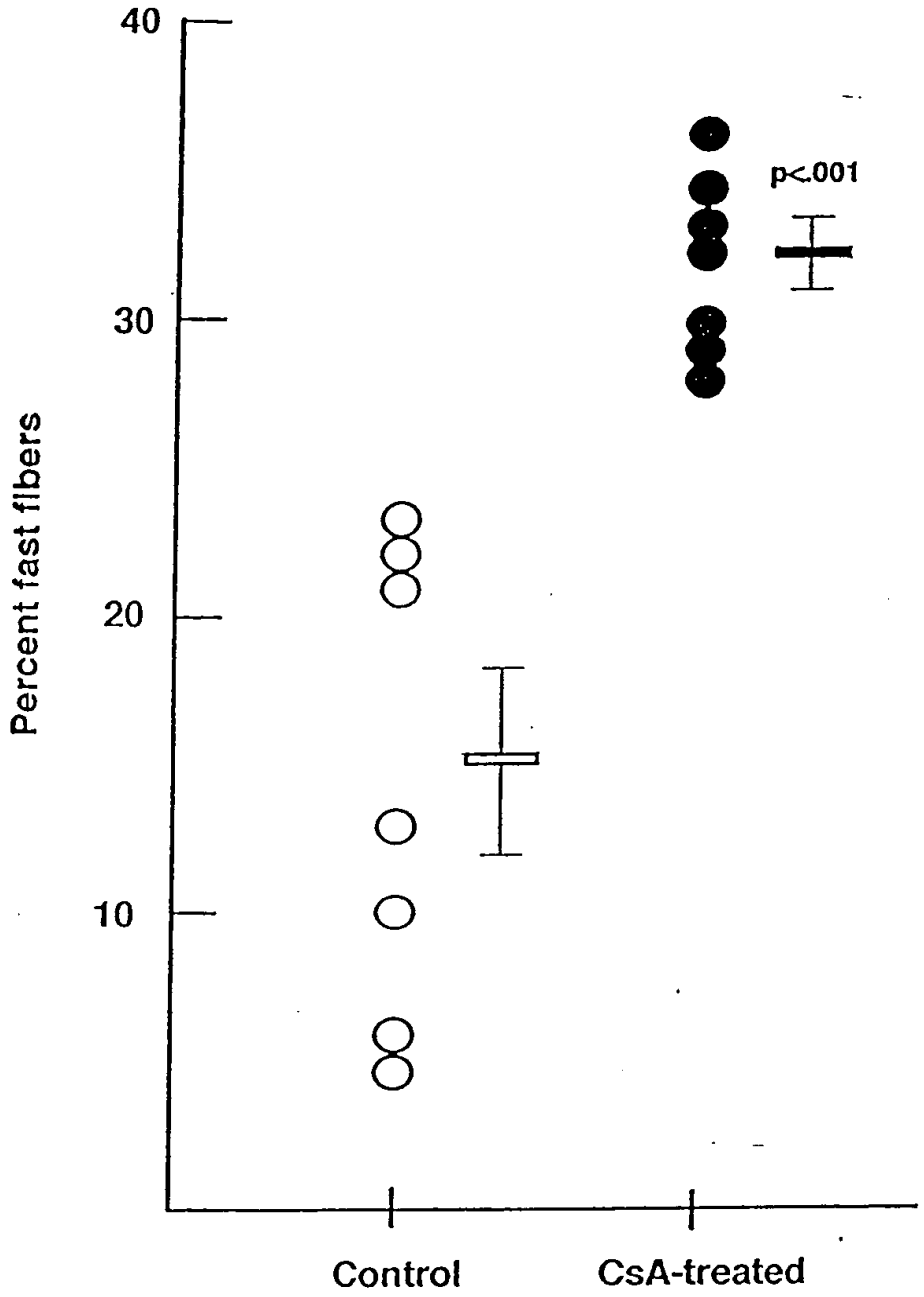


FIG. 4

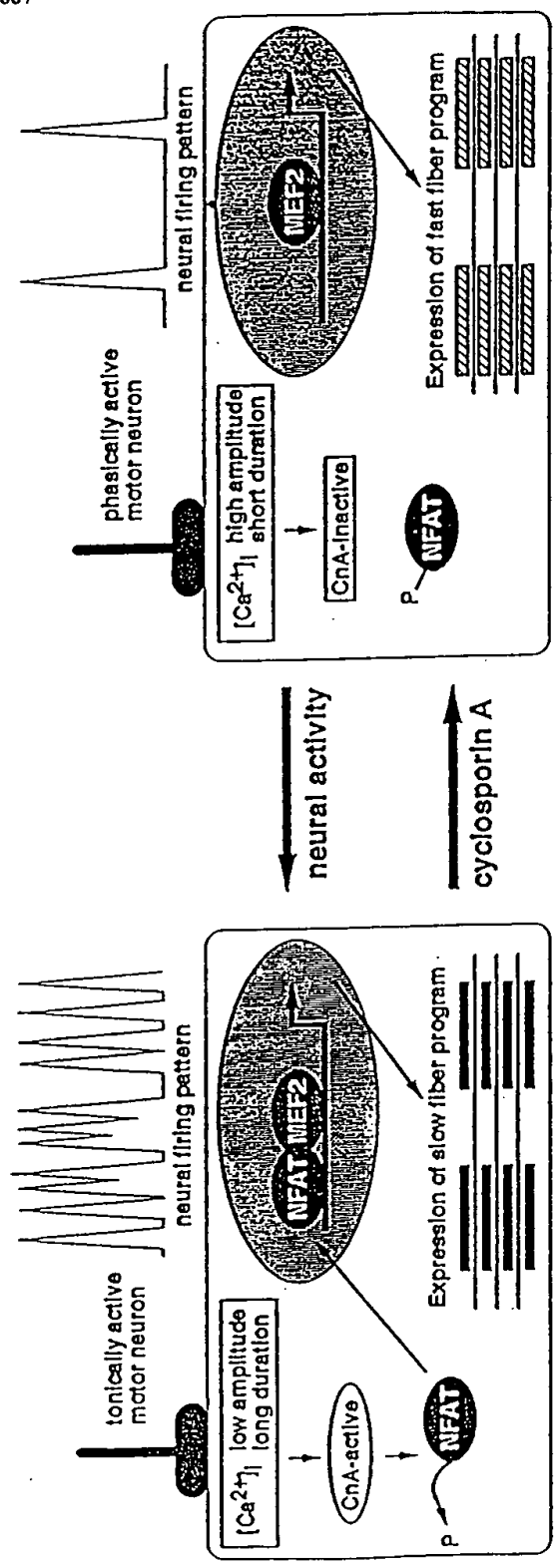
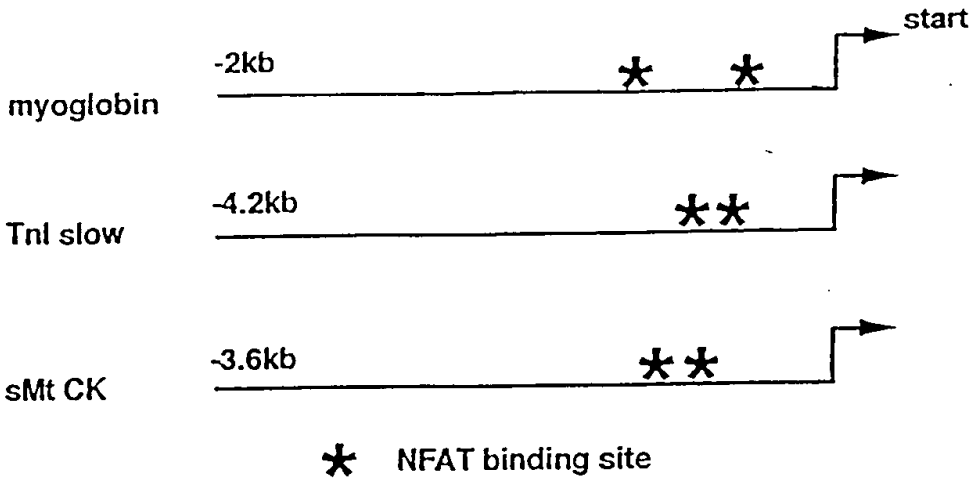

phasically active motor neuron
neural firing pattern
[Ca2+]i high amplitude short duration
CnA-Inactive
NFAT
P
MEF2
Expression of fast fiber program
fast fiber
neural activity
cyclosporin A
tonically active motor neuron
neural firing pattern
[Ca2+]i low amplitude long duration
CnA-active
NFAT
P
NFAT MEF2
Expression of slow fiber program
slow fiber

FIG. 5



gene	sequence
myoglobin	$^{-232}$TGGAAAGA $^{-690}$AGGAAATA
Tnl slow	$^{-639}$TGGAAACA $^{-738}$AGGAAACC
sMt CK	$^{-749}$TGGAAACT $^{-859}$AGGAAACT
NFAT consensus:	A T GGAAANA T C

FIG. 6A

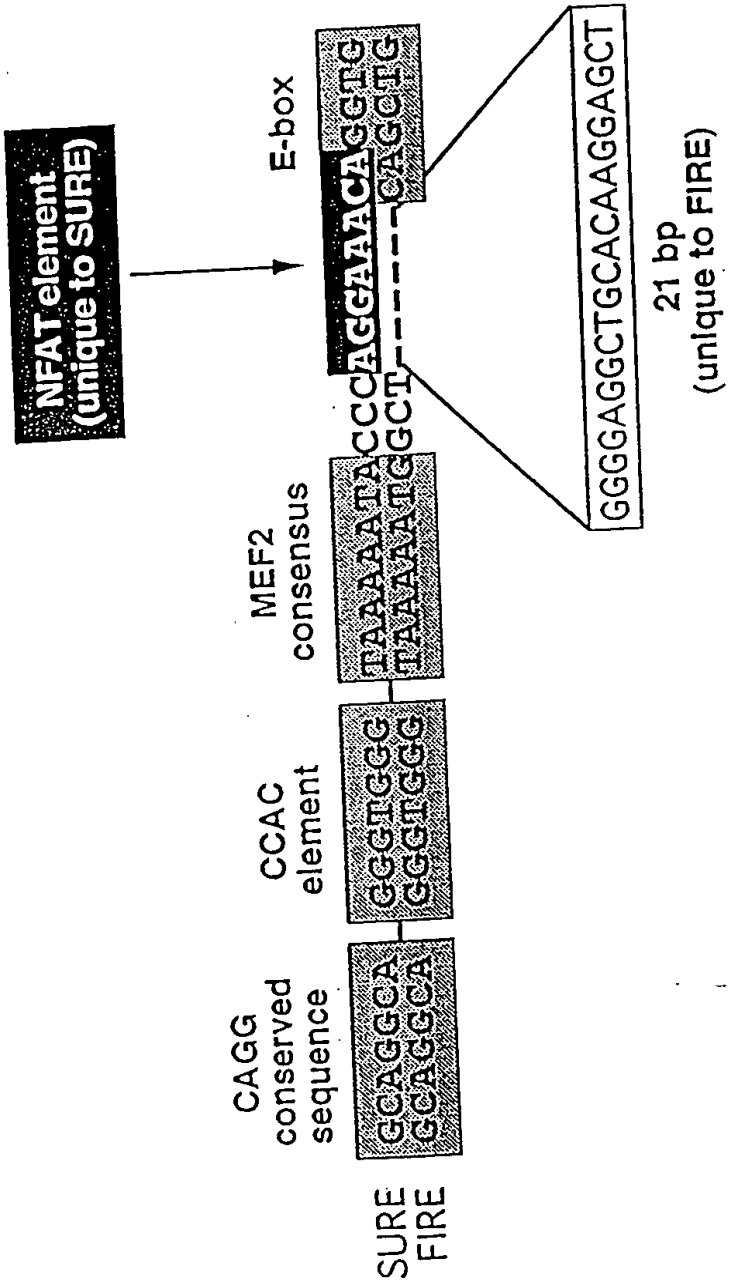

NFAT element (unique to SURE)
E-box
CAGGAAACAGGTG
CAGCTG
GGGGAGGCTGCACAAGGAGCT
21 bp (unique to FIRE)
MEF2 consensus
TAAAAATA
TAAATATG
CCAC element
GGGTGGG
GGGTGGG
CAGG conserved sequence
GCAGGCA
GCAGGCA
SURE
FIRE

FIG. 6B

SEQUENCE LISTING

<110> Williams, R. Sanders
Olson, Eric N.

<120> CALCINEURIN-DEPENDENT CONTROL OF SKELETAL MUSCLE FIBER TYPE

<130> UTXD:562PZ1

<140> Unknown
<141> 1999-08-13

<150> 60/096,631
<151> 1998-08-14

<160> 1

<170> PatentIn Ver. 2.0

<210> 1
<211> 21
<212> DNA
<213> Homo sapiens

<400> 1
aaccaggaaa taggatgccc t 21

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/18439

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 33887 A (UNIV JOHNS HOPKINS MED) 6 August 1998 (1998-08-06) the whole document ---	1-69
Y	WO 95 12979 A (UNIV SOUTHERN CALIFORNIA) 18 May 1995 (1995-05-18) the whole document ---	1-69
Y	US 5 352 595 A (TAPSCOTT STEPHEN J ET AL) 4 October 1994 (1994-10-04) the whole document --- -/--	1-69

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1999	23/11/1999

Name and mailing address of the ISA
European Patent Office, P.B 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer
Hillenbrand, G

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GROBET ET AL: "Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle" MAMMALIAN GENOME,US,NEW YORK, NY, vol. 9, no. 3, - March 1998 (1998-03) page 210-213 XP002085799 the whole document ---	1-69
Y	KAMBADUR ET AL: "Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle" GENOME RESEARCH,US,COLD SPRING HARBOR LABORATORY PRESS, vol. 7, no. 9, - September 1997 (1997-09) page 910-916 XP002085802 ISSN: 1088-9051 the whole document ---	1-69
P,X	WO 99 02667 A (GEORGES MICHEL ;GROBET LUC (BE); UNIV LIEGE (BE); PONCELET DOMINIQ) 21 January 1999 (1999-01-21) the whole document -----	1-69

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/18439

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9833887 A	06-08-1998	AU 6274298 A	25-08-1998
WO 9512979 A	18-05-1995	AU 1052795 A	29-05-1995
US 5352595 A	04-10-1994	NONE	
WO 9902667 A	21-01-1999	AU 8457198 A	08-02-1999

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B3

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 5/06

A1

(11) International Publication Number: WO 00/09667

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18439

(22) International Filing Date: 13 August 1999 (13.08.99)

(30) Priority Data:
60/096,631 14 August 1998 (14.08.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/096,631 (CON)
Filed on 14 August 1998 (14.08.98)

(71) Applicant *(for all designated States except US)*: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).

(71)(72) Applicants and Inventors: WILLIAMS, R., Sanders [US/US]; 3628 Mockingbird Lane, Dallas, TX 75205 (US). OLSON, Eric, N. [US/US]; 3700 Southwestern Boulevard, Dallas, TX 75225 (US).

(74) Agent: HIGHLANDER, Steven, L.; Arnold White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CALCINEURIN-DEPENDENT CONTROL OF SKELETAL MUSCLE FIBER TYPE

(57) Abstract

The present invention relates to skeletal muscle fiber composition. More particularly, the present invention defines the molecular events linking calcium stimulation to specialization of skeletal muscle fibers. More specifically, the present invention shows that Ca^{2+} stimulation of the slow fiber phenotype is mediated through a calcineurin-dependent pathway. Thus, the present invention provides methods and compositions for altering and/or regulating the phenotype of muscle. Further provided are methods for the detection of compounds having therapeutic activity toward regulating muscle fiber composition.

*(Referred to in PCT Gazette No. 25/2000, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DESCRIPTION

CALCINEURIN-DEPENDENT CONTROL OF SKELETAL MUSCLE FIBER TYPE

BACKGROUND OF THE INVENTION

This application claims priority to and specifically incoporates by reference, the content of U.S. Provisional Patent Application Serial No. 60/096,631, filed August 14, 1998. The entire text of the above–referenced disclosure is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of molecular biology and physiology. More particularly, it concerns the identification of calcineurin as a central mediator for muscle fiber phenotype.

2. Description of Related Art

There are many disorders in which the muscle fiber type of skeletal muscle plays a role. For example, progressive myonecrosis occurs with advancing age in individuals lacking dystrophin (Duchenne's muscular dystrophy) (Webster *et al.*, 1988). Patients with congestive heart failure, irrespective of the primary cause, exhibit loss of slow, oxidative myofibers in their skeletal muscles (Massie *et al.*, 1988; Sabbah *et al.*, 1993), an abnormality that contributes to exercise intolerance in these individuals. A decline in slow fibers also is observed as a result of prolonged inactivity or hypogravity (Caiozzo *et al.*, 1994), and the fiber composition of skeletal muscles influences insulin sensitivity (Kong *et al.*, 1994) and lipoprotein metabolism (Tikkanen *et al.*, 1996). Thus, the presence of a specific subtype of skeletal myofibers has a defined role in the pathophysiology of various muscular diseases.

Subtypes of skeletal myofibers of adult vertebrates differ markedly with respect to contractile physiology, metabolic capabilities, ultrastructural morphology, and susceptibility to fatigue. The physiological and clinical importance of myofiber specialization has been recognized for several decades, and many studies have identified sets of specific contractile proteins and enzymes of intermediary metabolism, the selective expression of which establishes this physiological and biochemical diversity among skeletal myocytes (Saltin and Gollnick, 1983; Booth and Baldwin, 1996; Schiaffino and Reggiani, 1996).

Fiber type-specific programs of gene expression can be detected at early stages of myogenic development in the embryo (DiMario *et al.*, 1993; Ontell *et al.*, 1993; Stockdale 1997), but remain plastic in adults, where they are subject to modification as a function of contractile load (*e.g.*, exercise training), hormonal shifts, or systemic diseases (Holloszy and Coyle, 1984; Williams and Neufer, 1996; Ianuzzo *et al.*, 1991; Massie *et al.*, 1988; Sabbah *et al.*, 1993).

A central role for motor nerve activity in determining skeletal muscle fiber composition was revealed by cross-innervation and electrical stimulation experiments, which demonstrated complete and reversible transformation of pre-existing myofibers by changing patterns of neuronal firing (Vrbova, 1963; Williams *et al.*, 1986; Pette and Vrbova, 1992). Specifically, brief bursts of neural activity, interspersed between long periods of neuronal quiescence, promote the acquisition of fast-twitch, glycolytic fiber characteristics. Conversely, extended periods of tonic motor nerve activity stimulate a shift to the slow-twitch, oxidative myofiber phenotype.

Neural stimulation provokes changes in the intracellular concentrations of several potential signaling molecules including calcium, cyclic AMP and nitric oxide, as well as immediate early gene products (c-fos) and molecular chaperones (hsp70) (Michel *et al.*, 1994; Neufer *et al.*, 1996; Williams and Neufer, 1996), but specific signaling pathways and regulatory molecules that link motor nerve activity to fiber type-specific gene expression have yet to be identified.

Tonic motor nerve activity at 10-15 Hz is characteristic of slow-twitch fibers (Hennig and Lomo, 1985) and results in a sustained elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$) within a concentration range between 100 and 300 nM (Chin and Allen, 1996), a pattern predicted to activate calcineurin. In fast myofibers, resting $[Ca^{2+}]_i$ is maintained at levels of only 50 nM (Westerblad and Allen, 1991), and the high amplitude (~1M) calcium transients evoked by motor nerve activity are predicted to be of insufficient duration to evoke calcineurin-stimulated signaling. Chronic stimulation at 10 Hz of the motor nerve innervating fast myofibers results in sustained elevations of $[Ca^{2+}]_i$ and promotes fast-to-slow fiber transformation (Williams *et al.*, 1986; Sreter *et al.*, 1987).

Calcineurin-dependent signaling mechanisms have been characterized extensively in the activation of cytokine gene expression in T and B lymphocytes responding to stimuli that elevate $[Ca^{2+}]_i$ (Rao *et al.*, 1997). Calcineurin and several NFAT isoforms are abundant in skeletal muscles (Hoey *et al.*, 1995), though target genes that respond to this pathway in skeletal myocytes have not been identified previously, and a specific role for calcineurin in the control of myofiber specialization has not been previously proposed.

The need to control specific fiber composition of skeletal muscle has significant implications. The elucidation of a signaling pathway linking motor nerve activity to selective changes in gene expression that establish diversity among myofibers will be particularly useful in ameliorating the pathological effects of various muscular diseases, and altering properties of skeletal muscle to prevent or treat systemic diseases such as diabetes and atherosclerosis. The present invention is directed to these and other deficiencies in the art relating to myofiber specialization.

SUMMARY OF THE INVENTION

The present invention provides a method of altering the phenotype of skeletal muscle tissue, comprising contacting the tissue with a modulator of calcineurin activity. In specific embodiments, altering the phenotype comprises an increase of the proportion fast fiber to slow fiber in the tissue. In alternative embodiments, altering the phenotype comprises an increase of the proportion slow fiber to fast fiber in the tissue. In particular aspects of the present invention, the phenotype comprises a change in the size of the cells of the tissue.

In particular embodiments, the muscle cells are derived from soleus, gastroenemius, quadriceps, tibialis anterior, pectordis, lattissimus dorsi, diaphragm, biceps, triceps, gluteus and tongue. Of course, these are exemplary muscle cells and the methods of the present invention may employ any muscle cell in which a fast to slow or slow to fast fiber composition transition is desired, such additional muscle cells are well known to those of skill in the art. In specific embodiments, the muscle cells are soleus muscle cells. The muscle cells may be derived from any animal including but not limited to human, murine, bovine, equine, porcine, ovine, canine, feline, rodent, avian or fish. In more specific embodiments the animal is a human.

In other embodiments, altering the phenotype comprises an increase in expression of fiber-type specific gene expression. More particularly, the fiber-type specific gene expression is specific to fast fiber cells. It is specifically contemplated that the gene expression comprises the expression of muscle creatinine kinase, fast myosin heavy chain, fast myosin light chain, fast troponin or parvalbumin. Alternatively, the fiber-type specific gene expression is specific to slow fiber cells. More particularly, the slow fiber specific gene expression comprises the expression of myoglobin, troponin I, slow myosin heavy chain, slow myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase.

It is particularly contemplated that the modulator is an inhibitor of calcineurin activity. More particularly, the inhibitor of calcineurin reduces the expression of calcineurin. In specific embodiments, the inhibitor of calcineurin is an agent that binds to and inactivates calcineurin. In an alternative embodiments, the inhibitor of calcineurin inhibits the interaction of calcineurin with an NFAT. In yet another embodiment, the agent that reduces the expression of calcineurin is an antisense construct. In those embodiments in which the agent binds to and inactivates calcineurin, such an agent may be an antibody or a small molecule inhibitor. More particularly, the antibody is a single chain antibody. In alternative embodiments, the antibody is a monoclonal antibody. Alternative particular embodiments contemplated that the inhibitor may be selected from the group consisting of cyclosporin, FK506, AP1510 and FK1012. Of course, these are exemplary inhibitors; it is contemplated that additional inhibitors derived from these compounds or acting through the same mechanisms of action as these compounds also will be useful in the context of the present invention. Such derivatives may be naturally occurring variants of these compound, may be produced through rational drug design based upon the structure of these inhibitors, or discovered from small molecule libraries.

In additional embodiments, the modulator may be a stimulator of calcineurin activity. Specifically, it is contemplated that the stimulator of calcineurin activity increases the expression of calcineurin. In other embodiments, the stimulator of calcineurin activity activates the calcineurin. More particularly, the activator of calcineurin activity may be calcium or calmodulin, or any other protein or molecule that acts in a manner to activate calcineurin activity, expression or function. In certain embodiments, the modulator of calcineurin activity increases the activity of an NFAT. In more particular embodiments, the increase in an NFAT

activity comprises stimulation of the dephosphorylation of an NFAT. In certain embodiments, the increase in an NFAT activity comprises increasing the expression of an NFAT. In other embodiments, the increase in an NFAT activity comprises contacting NFAT with an agent that activates the NFAT. In still further embodiments, the increase in an NFAT activity comprises increasing the interaction of NFAT with MEF2. Of course, it is contemplated that the increase in an NFAT activity also may comprises increasing the interaction of NFAT with additional or alternative transcription factors. It is specifically contemplated that the NFAT may be selected from the group consisting of NFAT1, NFAT2, NFAT3 and NFAT4. These are exemplary members of the NFAT family, it is understood that the present invention may employ any one or a combination of these or other NFATs that are found in muscle cells.

In particular embodiments, the modulator of calcineurin activity inhibits the activity of an NFAT. More particularly, inhibition of the activity of an NFAT comprises inhibiting the dephosphorylation of NFAT. In other embodiments, the inhibition of the activity of an NFAT comprises reducing the expression of NFAT. In still further embodiments, inhibition of the activity of an NFAT comprises contacting NFAT with an agent that binds to and inactivates NFAT. In additional embodiments, inhibition of the activity of an NFAT comprises inhibiting the interaction of NFAT with MEF2. In specific embodiments, the agent that reduces the expression of an NFAT is an antisense construct. In other embodiments, the agent that binds to and inactivates an NFAT is an antibody or a small molecule inhibitor.

Also provided herein is a method of transforming a fast muscle fiber to a slow muscle fiber comprising increasing the calcineurin activity in the fast muscle fiber. More specific embodiments contemplate that the calcineurin is encapsulated in a liposome. In alternative embodiments, it is contemplated that the method of transforming a fast fiber to a slow fiber comprises the steps of providing an expression construct comprising a first nucleic acid encoding an active calcineurin and a promoter functional in the muscle fibers, wherein the nucleic acid is under transcriptional control of the promoter; and contacting the expression construct with the fast muscle fiber in an amount effective to promote the activation of NFAT of the fiber; wherein activation of NFAT in the fast fiber promotes the transformation of fast fiber to slow fiber.

In particularly contemplated embodiments, the fast fiber is located within an animal. In certain other embodiments, the first nucleic acid is a cDNA or genomic DNA. In certain preferred embodiments, the first expression construct is selected from the group consisting of an adenovirus, an adeno-associated virus, a vaccinia virus a herpes virus and a lentivirus. In other embodiments, the promoter may be selected from the group consisting of CMV IE, SV40 IE, RSV, β-actin, tetracycline regulatable and ecdysone regulatable. In particular embodiments, the contacting may be effected by direct injection of a muscle containing the slow fiber with the expression construct. In additional specific embodiments, the contacting may comprise delivering the expression construct intravenously, subcutaneously, intramuscularly, or intraperitoneally to a muscle containing the fast fiber.

Also contemplated herein is a method of transforming a slow muscle fiber to a fast muscle fiber comprising inhibiting calcineurin activity in the slow muscle fiber. More specifically, the method comprises the steps of providing an expression construct comprising a first nucleic acid encoding a calcineurin gene positioned antisense to a promoter functional in the slow muscle fiber, wherein the nucleic acid is under transcriptional control of the promoter; and contacting the expression construct with the slow muscle fiber in an amount effective to decrease the calcineurin activity in the fiber; wherein the decrease in calcineurin activity in the slow fiber promotes the transformation of slow fiber to fast fiber. In specific embodiments, it is contemplated that the method comprises contacting the slow muscle fiber with cyclosporin, FK506, AP1510 and FK1012. In other particular embodiments, the method comprises inhibiting the interaction of NFAT with MEF2.

Another aspect of the present invention provides a method of screening for modulators of muscle fiber phenotype comprising the steps of providing a skeletal muscle cell expressing an NFAT and/or a MEF2 gene; contacting the cell with a candidate modulator; and monitoring the cell for a phenotype that is absent when the cell is not treated with the candidate modulator. In preferred embodiments, the cell is in an animal. In other embodiments, the cell is derived from a fast muscle cell line. In yet another alternative, the cell is derived from a slow muscle cell line. It is contemplated that the contacting may be performed *in vitro*. In preferred embodiments, the monitoring may comprise measuring the activity or expression of a fast fiber-specific gene. In other embodiments, the monitoring may comprise measuring the activity or expression of a slow fiber-specific gene. In alternative embodiments, the monitoring may

comprise measuring the size or mass of the cell. In preferred embodiments, the monitoring may comprise monitoring Ca^{++} response in the cell. Specifically, monitoring the Ca^{++} response may comprise monitoring Ca^{2+} dependent gene expression in the cell. In certain embodiments, the contacting may be performed *in vivo*. In particular embodiments, the candidate modulator may be an antisense construct, a small molecule library, an antibody or more particularly a single chain antibody.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Response of different promoters to forced expression of a constitutively active form of calcineurin (O'Keefe *et al.*, 1992) in cultured C2C12 myotubes or NIH 3T3 fibroblasts. Promoter-reporter plasmids were constructed to link the indicated promoters (CMV, cytomegalovirus; TATA, a minimal promoter consisting of the TATA element from the human hsp70 gene; MCK, a 4.8 kb 5' flanking region from the murine muscle creatine kinase gene; Tnl slow, a 4.2 kb 5' flanking region from the human slow fiber-specific troponin I gene; or myoglobin, a 2 kb 5' flanking region from the human myoglobin gene) to a firefly luciferase reporter gene. The response to activated calcineurin was calculated as the fold-change in luciferase activity induced by activated calcineurin above that measured following transfection of the empty vector alone, corrected for transfection efficiency (β-galactosidase activity). Cyclosporin A (CsA) was added to the culture medium at the indicated final concentrations.

Histograms depict mean values (± SE) from 4-8 independent transfections in each cell background.

FIG. 2A and FIG. 2B. Role of NFAT proteins in calcineurin-dependent transactivation. Activity of wild-type and mutated myoglobin (FIG. 2A) or troponin I slow (FIG. 2B) gene promoters in differentiated C2C12 cells as a function of increasing doses of the calcineurin expression plasmid. Consensus NFAT recognition motifs at the indicated positions relative to the transcriptional start sites (see FIG. 6) were altered (ΔNFAT) by site-directed mutagenesis, and transfections were performed as described in FIG. 1. Data points represent mean values of luciferase activity, corrected for transfection efficiency (β-galactosidase activity), from duplicate transfections in a representative experiment, and expressed as a percentage of native promoter activity after transfection with the indicated amounts of activated calcineurin expression plasmid (CMV-CnA*).

FIG. 3A and FIG. 3B. Upstream regulatory elements of the myoglobin gene participating in calcineurin-dependent transactivation. Data are presented as reporter gene expression (mean ± SE of 6 independent transfections) normalized to activity of a cotranfected CMV-lacZ plasmid (luminometer units ($x10^5$)/well (1.9×10^5 cells)). (FIG. 3A) Responses of native (Mb380) or mutated variants of a truncated segment (-373 to +7) of the human myoglobin gene promoter to activated calcineurin. Nucleotide substitutions were introduced into each of two upstream regulatory elements shown previously to be essential for muscle-specific promoter activity (Devlin *et al.*, 1989; Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995; Grayson *et al.*, 1998). These mutated promoters (MbΔA/T and-MbΔCCAC) are likewise defective for calcineurin-stimulated transactivation. (FIG. 3B) Responses to activated calcineurin of synthetic promoters constructed with various combinations of multimerized oligonucleotide cassettes representing protein binding motifs (CCAC, Sp1 binding site; A/T, MEF2 binding site; NRE, putative NFAT binding site; TATA, TBP binding site and core promoter) from the myoglobin promoter.

FIG. 4. Fiber composition of soleus muscles from intact rats treated with cyclosporin A. Circles represent individual animals (open, vehicle-treated; closed, cyclosporin A treated), and mean values in each group (± SE) are shown as horizontal lines. The difference in group means was highly significant ($p<.001$ by unpaired Student's t test).

FIG. 5. Model for a calcineurin-dependent pathway linking specific patterns of motor nerve activity to distinct programs of gene expression that establish phenotypic differences between slow and fast myofibers. MEF2 is shown to represent the requirement for collaboration between activated NFAT proteins and muscle-restricted transcription factors in slow fiber-specific gene transcription, but other proteins (not shown) also are likely to participate.

FIG. 6A and FIG. 6B. NFAT consensus binding sequences are present within transcriptional control regions previously shown to direct transcription selectively in slow-oxidative myofibers (Parsons *et al.*, 1993; Levitt *et al.*, 1995; Qin *et al.*, 1997). (FIG. 6A) Consensus NFAT binding motifs in myoglobin, troponin I slow (TnI slow), and sarcomeric mitochondrial creatine kinase (sMtCK) promoters. (FIG. 6B) Conserved sequence blocks (CAGG, CCAC, MEF2, and E box) common to a Slow fiber-specific Upstream Regulatory Element (SURE) from the rat troponin I slow gene and a Fast fiber-specific Intronic Regulatory Element (FIRE) from the quail troponin I fast gene (Nakayama *et al.*, 1996). A predicted NFAT response element (darkly shaded) overlapping with an E-Box (MyoD binding site) and adjacent to a MEF-2 binding site.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Fiber type of skeletal muscle plays a role in numerous disorders including progressive myonecrosis (Duchenne's muscular dystrophy) (Webster *et al.*, 1988), congestive heart failure, (Massie *et al.*, 1988; Sabbah *et al.*, 1993), decline in slow fibers observed as a result of prolonged inactivity or hypogravity (Caiozzo *et al.*, 1994), and the fiber composition of skeletal muscles influences insulin sensitivity (Kong *et al.*, 1994) and lipoprotein metabolism and atherosclerosis (Tikkanen *et al.*, 1996).

There is a well-established association between motor nerve activity and specialized fiber characteristics that has been described extensively in the literature on muscle plasticity (Michel *et al.*, 1994; Neufer *et al.*, 1996; Williams and Neufer, 1996). Fast-to-slow fiber transformation is evoked by increased motor nerve activity, stimulated by cross-innervation, electrical pacing or exercise training. Slow-to-fast fiber transformation occurs as a

consequence of decreased motor nerve activity, resulting from cross-innervation, certain disease states, hypogravity, or physical inactivity. The present invention describes methods and compositions for controlling these transformations.

1. The Present Invention

Slow- and fast-twitch myofibers of adult skeletal muscles express unique sets of muscle-specific genes, and these distinctive programs of gene expression are controlled by variations in motor neuron activity. It is well established that, as a consequence of more frequent neural stimulation, slow fibers maintain higher levels of intracellular free calcium than fast fibers, but the mechanisms by which calcium may function as a messenger linking nerve activity to changes in gene expression in skeletal muscle were unknown. Here, fiber type-specific gene expression in skeletal muscles is shown to be controlled by a signaling pathway that involves calcineurin, a cyclosporin-sensitive, calcium-regulated serine/threonine phosphatase. Activation of calcineurin in skeletal myocytes selectively up-regulates slow fiber-specific gene promoters. Conversely, inhibition of calcineurin activity by administration of cyclosporin A (CsA) to intact animals promotes slow-to-fast fiber transformation. Transcriptional activation of slow fiber-specific transcription appears to be mediated by a combinatorial mechanism involving proteins of the NFAT and MEF2 families. The present invention thus identifies a molecular mechanism by which different patterns of motor nerve activity promote selective changes in gene expression to establish the specialized characteristics of slow and fast myofibers.

The results described herein show a molecular model, not previously considered, to explain how motor nerve activity controls programs of gene expression that define fast and slow subtypes of skeletal myofibers (FIG. 5). The model proposes that tonic motor nerve activity, characteristic of nerves innervating slow muscles, sustains $[Ca^{2+}]_i$ at levels sufficient to activate the calcineurin-NFAT pathway. The protein phosphatase activity of calcineurin leads to dephosphorylation and nuclear localization of NFAT proteins. In the nucleus, NFAT proteins bind DNA in conjunction with other transcriptional regulators, including (but not limited to) MEF2, binding sites for which are clustered in promoter/enhancer regions controlling transcription of genes encoding proteins of the slow fiber program. In fast fibers, high amplitude calcium transients stimulated by infrequent, phasic firing of the motor nerve are of insufficient duration to maintain calcineurin in the active state, so NFAT proteins remain

phosphorylated and are excluded from the nucleus. When NFAT proteins are unavailable for DNA binding and protein-protein interactions at target promoters, the slow fiber-specific program is down-regulated, and genes encoding fast fiber-specific proteins are transcribed.

Using the insights gained herein, the present invention is directed to methods of altering the phenotype of skeletal muscle tissue, by contacting the tissue with a modulator of calcineurin activity. Thus, the method of the present invention may be used to increase the proportion of slow fiber to fast fiber in said tissue. Conversely, the methods may be used to increase of the proportion fast fiber to slow fiber in the tissue. Additional embodiments contemplate methods of screening for modulators of muscle fiber phenotype. These and other aspects of the present invention are discussed in further detail herein below.

2. A Calcineurin-Dependent Pathway in Muscle Fiber

It is known that $[Ca^{2+}]i$ levels play a role in the muscle fiber type characteristics of skeletal muscle. However, the possibility that calcineurin might be involved in skeletal muscle fiber type specialization has not been previously investigated. The present invention describes calcineurin-dependent transformation from fast to slow muscle fiber by transactivation of slow fiber-specific gene promoters, whereas inhibition calcineurin leads to a preponderance of fast fiber type phenotype. This pathway is depicted in FIG. 5. The individual components of this pathway, as they relate to muscle fiber composition in skeletal muscle, are discussed in further detail herein below.

a. Calcineurin

Calcineurin is a ubiquitously expressed serine/threonine phosphatase that exists as a heterodimer, comprised of a 59 kD calmodulin-binding catalytic A subunit and a 19 kD Ca^{2+}-binding regulatory B subunit (Stemmer and Klee, 1994; Su *et al.*, 1995). Calcineurin is activated by a sustained Ca^{2+} plateau and is insensitive to transient Ca^{2+} fluxes as occur in response (Dolmetsch *et al.*, 1997). Activation of calcineurin is mediated by binding of Ca^{2+} and calmodulin to the regulatory and catalytic subunits, respectively.

Calcineurin-dependent signaling mechanisms have been characterized extensively in the activation of cytokine gene expression in T and B lymphocytes responding to stimuli that elevate intracellular free calcium concentration ($[Ca^{2+}]_i$) (Rao *et al.*, 1997). Binding of calcium

to a calmodulin-calcineurin complex stimulates serine/threonine phosphatase activity of calcineurin, the major substrates of which are NFAT (Nuclear Factor of Activated T cells) transcription factors.

Dephosphorylation of NFATs by calcineurin promotes their translocation from the cytoplasm to the nucleus, where they bind a cognate nucleotide recognition sequence (Rao *et al.*, 1997) and stimulate transcription of target genes that, in lymphocytes, include hematopoietic growth factors (*e.g.*, GM-CSF) and inflammatory cytokines (*e.g.*, IL-2). Recent studies demonstrate that calcineurin activity and the resulting nuclear translocation of NFAT are insensitive to transient, high amplitude oscillations in $[Ca^{2+}]_i$ that activate other calcium-dependent events (*e.g.*, NF-κB or c-Jun N-terminal kinase). Rather, the calcineurin-NFAT pathway responds preferentially to sustained, low amplitude elevations of $[Ca^{2+}]_i$ (Timmerman *et al.*, 1996; Dolmetsch *et al.*, 1997). This ability of a calcineurin-dependent signaling pathway to discriminate between different patterns in the amplitude and duration of changes in $[Ca^{2+}]_i$, and the observation that there are differences in the $[Ca^{2+}]$ among specialized myofiber subtypes, provide the backdrop for the present invention.

The present invention demonstrates that forced expression of constitutively active calcineurin selectively transactivates promoters from two genes that are expressed preferentially in slow versus fast skeletal myofibers. Thus, downstream effectors of a calcineurin-regulated signaling pathway are present and capable of transducing the signal in a muscle cell background, and transcriptional regulatory elements capable of receiving the signal are present within genes representative of the slow fiber program. Specific effector molecules appear to include NFAT proteins, since consensus NFAT binding motifs contained within slow fiber-specific promoters participate in the response to activated calcineurin, and several NFAT isoforms are expressed in skeletal muscle (Hoey *et al.*, 1995).

Further, the data described herein show that DNA binding of NFAT proteins is not sufficient to transduce the calcineurin-generated signal in skeletal myocytes. Rather, NFAT transcription factors collaborate with MEF2 and other transcriptional regulatory proteins, the correct combination of which is present within differentiated myotubes, but absent from undifferentiated myoblasts or fibroblasts. Previous studies of calcineurin-stimulated transactivation of cytokine gene promoters in T cells, where AP-1 cooperates with NFAT in

both DNA binding and transactivation (reviewed by Rao *et al.*, 1997), provide a precedent for synergistic combinatorial interactions between NFAT proteins and heterologous transcription factors.

The organization of transcriptional control regions that confer fiber type-specific expression (FIG. 6) is consistent with this viewpoint. Consensus NFAT binding sequences are conserved in the 5' flanking region of myoglobin and TnIs genes from all vertebrate species in which promoter sequences are available, and in other slow fiber-specific enhancers (FIG. 6A). Moreover, two studies that have mapped fiber type-specific enhancers at the highest resolution. Buonanno and colleagues (Nakayama *et al.*, 1996) identified a 128-bp element from the rat TnIs gene (Slow Upstream Regulatory Element (SURE)) that confers slow fiber-specific transcription in transgenic mice, and a 144-bp element (Fast Intronic Regulatory Element (FIRE)) that directs fast fiber-specific expression of a different isoform of troponin I (TnIf).

The functionally distinctive SURE and FIRE elements contain similar or identical CAGG, CCAC, MEF2, and E box motifs (FIG. 6B), as found in many muscle-specific genes, so the basis for their reciprocal functions in specialized subtypes of myofibers has not been apparent. Examination of the SURE and FIRE elements in light of the observations described herein reveals a consensus NFAT recognition motif in the TnIs SURE element that is absent from the TnIf FIRE element. In the sarcomeric mitochondrial creatine kinase (sMtCK) gene, a 160-bp upstream element was shown to direct fiber type-specific gene expression in transgenic mice (Qin *et al.*, 1997). The sMtCK gene is expressed preferentially in slow, oxidative myofibers, in contrast to the MCK isoform (fast fiber specific) that was studied herein (FIG. 1). Like the myoglobin and TnIs gene enhancers, this sMtCK enhancer includes NFAT recognition motifs (FIG. 6A).

Furthermore, inhibition of calcineurin phosphatase activity in intact animals by administration of cyclosporin A leads to down-regulation of slow and induction of fast fiber type-specific markers. Methods and compositions for modulating this calcineurin-dependent effect in muscle fibers are presented herein below

b. Interaction between NFAT and Transcription Factors

Several forms of nuclear factor of activated T-cells (NFAT) are known to those of skill in the art, including NFAT1, NFAT2, NFATc, NFAT3, NFAT4a, NFAT4b and NFAT4c. Particular human NFATs are described in detail in U.S. Patent Number 5,708,158 (specifically incorporated herein by reference in its entirety). Additional information regarding the molecular interactions of NFAT proteins is provided by Nolan (1994). Northrop *et al.*, describes the cloning of a cDNA encoding human NFATc (1994). The cloning of a fragment of a gene encoding a murine NFAT1 also has been described (McCaffrey *et al.*, 1993)

These factors bind the consensus DNA sequence GGAAAAT as monomers or dimers through a Rel homology domain (RHD) (Rooney *et al.*, 1994; Hoey *et al.*, 1995). Three of the NFAT genes are restricted in their expression to T-cells and skeletal muscle, whereas NFAT3 is expressed in a variety of tissues including the heart (Hoey *et al.*, 1995). For additional disclosure regarding NFAT proteins the skilled artisan is referred to U. S. Patent 5,708,158.

Functional NFAT binding sites have been found in the promoters or enhancers of several different cytokine genes including IL-2, IL-4, IL-3, GM-CSF, and TNF-α and are often located next to AP-1 binding sites, which are recognized by members of the fos and jun families of transcription factors. Typically, the AP-1 binding sites adjacent to NFAT sites are low affinity sites, and AP-1 proteins cannot bind them independently. However, many NFAT and AP-1 protein combinations are capable of cooperatively binding to DNA. Furthermore, cell-type specificity of cytokine gene transcription is often controlled, at least in part, by the combinations of NFAT and AP-1 proteins present in those cells. For example, there are different classes of T cells that secrete different sets of cytokines: *e.g.*, TH1 cells produce IL-2 and IFN-γ, while TH2 cells produce IL-4, IL-5, and IL-6. NFAT binding sites are involved in the regulation of both TH1 and TH2 cytokines. Further, differential expression of the cytokine gene in T cell subsets is controlled the combinatorial interactions of NFAT and AP-1 proteins. Thus, NFAT has the ability to interact with additional factors in order to cooperatively bind DNA.

Recently, a calcineurin-dependent transcriptional pathway was shown to promote hypertrophic growth of the heart (Molkentin *et al.*, 1998). In cardiac myocytes, this pathway was shown to involve collaborative interactions between activated NFAT proteins and GATA4,

a cardiac-restricted transcription factor not present in skeletal muscle. Cardiomyocytes express several isoforms of MEF2, and many of the same genes that exhibit slow, oxidative fiber type-specific expression in skeletal muscle are transcriptionally active in cardiac myocytes (*e.g.*, myoglobin or sMtCK (Parsons *et al.*, 1993; Levitt *et al.*, 1995; Qin *et al.*, 1997).

Given the ability of NFAT factors to mediate changes in gene expression in response to Ca^{2+} signaling in T cells, and of cardiac gene expression, the inventors results presented herein suggest a mechanism for coupling calcineurin signaling to muscle fiber specific gene transcription.

c. Inhibitors of Calcineurin

Cyclosporin (CsA) and FK-506, bind the immunophilins cyclophilin and FK-506-binding protein (FKBP12), respectively, forming complexes that bind the calcineurin catalytic subunit and inhibit its activity. The results presented herein show that CsA and FK-506 inhibit calcineurin activity in intact animals and this block in calcineurin activity promotes slow-to-fast fiber transformation. Of note, it is known that hypertrophic agonists in heart muscle act by elevating intracellular Ca^{++}, which results in activation of the PKC and MAP kinase signaling pathways (Sadoshima and Izumo, 1993a, 1993b; Kudoh *et al.*, 1997; Yamazaki *et al.*, 1997; Zou *et al.*, 1996). CsA does not interfere with early signaling events at the cell membrane, such as PI turnover, Ca^{++} mobilization, or PKC activation (Emmel *et al.*, 1989).

In the context of the present invention, inhibitors of calcineurin activity have been shown to promote the transition between slow twitch fiber to fast fiber phenotype. This important observation answers why pharmacological blockade of calcineurin signaling in rats reduces the capacity for endurance exercise and diminishes peak rates of oxidative phosphorylation in mitochondria isolated from skeletal muscles (Mercier *et al.*, 1995). Clearly, the present results have a significant clinical relevance, revealing a need to modify skeletal muscle physiology and exercise performance in patients receiving CsA and FK506 and derivatives thereof in immunosuppressant drugs in current clinical use.

In specific embodiments of the present invention, it may be desirable to increase the proportion of fast fiber content in a slow muscle fiber population. Given the teachings of the present invention, such an effect may be achieved by the application of CsA, FK506, AP1510,

FK1012, any derivative thereof or any additional immunosuppressant that acts by inhibiting calcineurin expression, activity or function.

d. Muscle Fiber Type-Specific Genes

The present invention provides methods and compositions for altering the phenotype of a particular muscle fiber. The phenotypic properties of slow- and fast-twitch myofibers are determined by the selective transcription of genes coding for contractile proteins and metabolic enzymes in these muscles. Genes associated with fast fibers include, but are not limited to, muscle creatinine kinase, fast myosin heavy chain, (Hoh, 1992), fast myosin light chain (Jostarndt *et al.*, 1996; Rao *et al.*, 1996), fast troponin (Nakayama *et al.*, 1996; Briggs and Schachat, 1996) and parvalbumin (Nishida *et al.*, 1997). Genes associated with slow fibers include but are not limited to myoglobin (Williams *et al.*, 1997; Shen *et al.*, 1996), troponin I (Matsumoto *et al.*, 1997; Nakayama *et al.*, 1996; Levitt *et al.*, 1995), slow myosin heavy chain (Hoh, 1992; Mair *et al.*, 1992), slow myosin light chain (Jostarndt *et al.*,1996), mitochondrial proteins (Howlett and Willis, 1998; Delp *et al.*, 1997; Ogata and Yamasaki, 1997; Nakano *et al.*, 1997) GLUT4 (Marette *et al.*, 1992; Kong *et al.*, 1994; Ivy, 1996), or lipoprotein lipase (Borensztajn *et al.*, 1975; Kaciuba-Uscilko *et al.*, 1980; Mackie *et al.*, 1980; Tikkanen *et al.*, 1996). The genes specific for the slow muscle fiber phenotype possess binding sites for NFAT. Additionally, these genes may possess binding sites for transcription factors such as MEF2 and the like. The references above (each specifically incorporated herein by reference) describe the protein compositions of slow and fast twitch muscles. However, it is understood that these are exemplary in nature, additional fiber specific genes are well known to those of skill in the art and are contemplated for use in conjunction with the present invention.

3. Modulating Fiber Type Composition

Although the connection between $[Ca^{2+}]i$ and muscle tonic activity is well established, the present invention provides the first evidence of a calcineurin mediated pathway for the specialization of muscle fiber type. Essentially, the calcineurin is found to activate cytoplasmic NFAT by dephosphorylation. The dephosphorylated NFAT is translocated into the nucleus where it interacts with MEF2 and/or other transcription factors and upregulates the genes specific for slow fiber type muscle (*e.g.*, myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase).

Thus, in a particular embodiment of the present invention, there are provided methods of altering the phenotype of skeletal muscle tissue, by contacting the tissue with a modulator of calcineurin activity. These methods exploit the inventors' observation, described in detail herein, that calcinuerin, presumably through the involvement of one or more NFAT proteins, appears to up-regulate the expression of genes involved in the slow fiber muscle type, whereas inhibition of calcineurin up-regulates the expression of fast muscle fiber type.

At its most basic, the modulator of calcineurin activity will increase or potentiate the effect of calcineurin. This will function by increasing the *in vivo* activity of NFAT in the muscle fiber of an individual in need of slow muscle fiber type. Such a modulator will be useful in conditions presenting progressive myonecrosis, *e.g.*, Duchenne's muscular dystrophy and other diseases characterized by myodegeneration, myonecrosis and the like. This may be accomplished by one of several different mechanisms. First, one may increase the expression of calcineurin. Second, one may directly increase the function of the calcineurin protein by providing an agent activates the calcineurin protein, *e.g.*, calmodulin, Ca^{2+} and the like. And third, one may indirectly potentiate the effect of calcineurin by increasing the activity or expression of with one or more the NFAT targets of calcineurin. Alternatively, the effect may be further downstream of NFAT in which the activity of a transcription factor, such as a MEF2 or a gene influenced by the interaction of MEF2 and NFAT, such as myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase is increased.

In other embodiments, it may prove useful to increase the proportion of fast fiber in a particular muscle fiber. This embodiment will entail the inhibition of calcineurin activity. Such inhibition may be achieved by blocking the expression of calcineurin and/or the substrate for calcineurin activity (*i.e.*, NFAT). A second alternative would be to contact the muscle tissue with a one may directly decrease the function of the calcineurin protein by providing an agent inactivates the calcineurin protein, *e.g.*, cyclosporin A, FK506 and the like.

a. Increasing Slow Fiber Proportion

It has been demonstrated herein that in skeletal muscle fibers, the presence of an activated calcineurin dephosphorylates NFAT which, in turn, translocates to the nucleus where the dephosphorylated NFAT interacts with MEF2. The NFAT/MEF2 complex activates the

transcription of slow fiber specific genes including, but not limited to, myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase. Thus, it is envisioned that the phenotype of a particular muscle fiber may be altered by providing to the muscle fiber an activated form of calcineurin.

In other embodiments, an approach to alter the phenotype simply involves is the provision of a calcineurin polypeptide, active fragments, synthetic peptides, mimetics or other analogs thereof. The protein may be produced by recombinant expression means or, if small enough, generated by an automated peptide synthesizer. Formulations would be selected based on the route of administration and purpose including, but not limited to, liposomal formulations and classic pharmaceutical preparations.

In yet another alternative, the present invention may be used to alter the phenotype of a fast muscle fiber cell by providing to the cell a stimulator of calcineurin activity. Stimulators of calcineurin include Ca^{2+} and calmodulin. Further, the present invention provides details of method of identifying additional modulators of muscle fiber phenotype herein below.

As the present invention shows that calcineurin dephosphorylates NFAT in muscle fibers, and it is the dephosphorylated NFAT that mediates the effects of calcineurin and promotes the specialization of muscle fibers into slow fibers, it may prove useful to provide dephosphorylated NFAT to a muscle fiber. The NFAT may be provided as a protein composition or may alternatively be provided as an expression construct as described herein below.

b. Increasing Fast Fiber Proportion

In order to increase the proportion of fast fiber content in a particular composition, it will be necessary to block, inhibit or otherwise abrogate the action of calcineurin. Methods for this embodiment of the invention are outlined herein below.

i. Blocking Expression of Calcineurin and/or NFAT

The most direct method for blocking protein expression is via antisense technology. The term "antisense" is intended to refer to polynucleotide molecules complementary to a portion of a given RNA (*e.g.*, calcineurin, NFAT1, NFAT2, NFAT3, NFAT4) or the DNAs corresponding

thereto. "Complementary" polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs for the present invention will include regions complementary to the mRNA start site. One can readily test such constructs simply by testing the constructs *in vitro* to determine whether levels of the target protein are affected. Similarly, detrimental non-specific inhibition of protein synthesis also can be measured by determining target cell viability *in vitro*.

As used herein, the terms "complementary" or "antisense" mean polynucleotides that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen nucleotides out of fifteen. Naturally, sequences which are "completely complementary" will be sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a

non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

The polynucleotides according to the present invention may encode a calcineurin or NFAT gene or a portion of those genes that is sufficient to effect antisense inhibition of protein expression. The polynucleotides may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In other embodiments, however, the polynucleotides may be complementary DNA (cDNA). cDNA is DNA prepared using messenger RNA (mRNA) as template. Thus, a cDNA does not contain any interrupted coding sequences and usually contains almost exclusively the coding region(s) for the corresponding protein. In other embodiments, the antisense polynucleotide may be produced synthetically.

It may be advantageous to combine portions of the genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

The DNA and protein sequences for human NFAT family members have been published and are disclosed in U.S. Patent 5 ,708,158. It is contemplated that natural variants of exist that have different sequences than those disclosed herein. Thus, the present invention is not limited to use of the provided polynucleotide sequence for calcineurin or NFAT but, rather, includes use of any naturally-occurring variants. Depending on the particular sequence of such variants, they may provide additional advantages in terms of target selectivity, *i.e.*, avoid unwanted antisense inhibition of related transcripts. The present invention also encompasses chemically synthesized mutants of these sequences.

Although the antisense sequences may be full length genomic or cDNA copies, or large fragments thereof, they also may be shorter fragments, or "oligonucleotides," defined herein as polynucleotides of 50 or less bases. Although shorter oligomers (8-20) are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of base-pairing. For example, both binding affinity and sequence specificity of an oligonucleotide to its complementary target increase with increasing length. It is contemplated that oligonucleotides

of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50 base pairs will be used. While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression.

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" is refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in both DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression vector encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense polynucleotide. Ribozyme sequences also may be modified in much the same way as described for antisense polynucleotide. For example, one could incorporate non-Watson-Crick bases, or make mixed RNA/DNA oligonucleotides, or modify the phosphodiester backbone, or modify the 2'-hydroxy in the ribose sugar group of the RNA.

Alternatively, the antisense oligo- and polynucleotides according to the present invention may be provided as RNA via transcription from expression constructs that carry nucleic acids encoding the oligo- or polynucleotides. Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid encoding an antisense product in which part or all of the nucleic acid sequence is capable of being transcribed. Typical expression vectors include bacterial plasmids or phage, such as any of the pUC or Bluescript™ plasmid series or, as discussed further below, viral vectors adapted for use in eukaryotic cells.

In preferred embodiments, the nucleic acid encodes an antisense oligo- or polynucleotide is placed in a replicable cloning vehicle that supports expression of the antisense molecule with

cis-acting transcriptional and translational signals. The expression constructs will comprise the gene in question and various regulatory elements as described herein below.

ii. Blocking Function of Calcineurin

In another embodiment, it may be desirable to block the function of a calcineurin and/or NFAT polypeptide rather than inhibit expression. This can be accomplished by use of organochemical compositions that interfere with the function of the protein, by use of an antibody that blocks an active site or binding site on calcinuerin or NFAT, or by use of a molecule that mimics a target of calcineurin (*i.e.*, NFAT) or of NFAT (*i.e.*, MEF2, GATA4 and the like).

With respect to organochemical inhibitors, such compounds may be identified in standard screening assays. For example, it is known that calcineurin bind to NFAT. Various candidate substances can be contacted with calcineurin followed by further determination of the ability of treated calcineurin to bind NFAT. Alternatively, given the knowledge that NFAT is activated as a result of dephosphorylation by calcineurin, and it is this activation that produces the upregulation of the slow fiber phenotype, it now is possible to provide an activator or inhibitor *in vivo* to an appropriate animal, *e.g.*, a mouse, and look for decreased muscle growth. Once identified, such a modulator may be used to stimulate or inhibit calcineurin and/or NFAT3 function in a therapeutic context.

With respect to antibodies, it should be noted that not all antibodies are expected to have the same functional effects on their targets. This stems both from the differing specificities of antibodies and their character, *i.e.*, their isotype. Thus, it will be useful to generate a number of different monoclonal and polyclonal preparations against calcineurin. It also may prove useful to generate anti-idiotypic antibodies to anti-calcineurin antibodies. These compounds may be used as probes for calcineurin binding partners such as members of the NFAT family. Additional similar antibodies directed against NFAT may be used to identify NFAT putative binding partners, such as MEF2 and other nuclear transcriptional factors.

The methods by which antibodies are generated are well known to those of skill in the art, and are detailed elsewhere in the specification. Again, antibodies that bind to calcineurin

may be screened for other functional attributes, *e.g.*, blocking of NFAT binding, blocking of dephosphorylation of NFAT *etc.* in *in vitro* assays prior to their implementation *in vivo*.

A particularly useful antibody for blocking the action of a given protein is a single chain antibody. Methods for the production of single-chain antibodies are well known to those of skill in the art. The skilled artisan is referred to U.S. Patent 5,359,046, (incorporated herein by reference) for such methods. A single chain antibody, preferred for the present invention, is created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (Fvs) in which the C-terminus of one variable domain is tethered to the N-terminus of the other via a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk *et al.*, 1990; Chaudhary *et al.*, 1990). These Fvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody.

With respect to inhibitors that mimic calcineurin or NFAT targets, the use of mimetics provides one example of custom designed molecules. Such molecules may be small molecules that specifically stimulate or inhibit calcineurin or NFAT protein activity or alternatively, stimulate or inhibit binding to MEF2. Such molecules may be sterically similar to the actual target compounds, at least in key portions of the target's structure and or organochemical in structure. Alternatively, these inhibitors may be peptidyl compounds, these are called peptidomimetics. Peptide mimetics are peptide-containing molecules which mimic elements of protein secondary structure. See, for example, Johnson *et al.*, (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of ligand and receptor. An exemplary peptide mimetic of the present invention would, when administered to a subject, bind to NFAT in a manner analogous to MEF2.

Successful applications of the peptide mimetic concept have thus, far focused on mimetics of β-turns within proteins, which are known to be highly antigenic. Likely β-turn structures within an antigen of the invention can be predicted by computer-based algorithms as discussed above. Once the component amino acids of the turn are determined, mimetics can be constructed to

achieve a similar spatial orientation of the essential elements of the amino acid side chains, as discussed in Johnson *et al.*, (1993).

iii. Blocking of a Target

As discussed above, one of the benefits of the present invention is the identification of targets upon which calcineurin acts. These targets may be binding partners such as NFAT. The NFAT in turn binds to MEF2 or other genes that are upregulated by an activated NFAT interaction with MEF2, such as myoglobin, troponin I, myosin heavy chain, myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase. In order to prevent calcineurin from interacting with these targets, one may take a variety of different approaches. For example, one may generate antibodies against the target and then provide the antibodies to the subject in question, thereby blocking access of calcineurin to the target molecule. Equally antibodies against NFAT will prevent the binding of NFAT to MEF2 and therefore prevent the upregulation of slow twitch specific genes.

In yet another embodiment, antisense methodologies may be employed in order to inhibit the interaction of NFAT with its target, seeing as the NFAT binding partner is a DNA molecule. Alternatively, one may design a polypeptide or peptide mimetic that is capable of interacting with the NFAT target in the same fashion as NFAT, but without any NFAT-like effect on the target.

In a preferred embodiment, the present invention will provide an agent that binds competitively to MEF2. In a more preferred embodiment, the agent will have an even greater affinity for the MEF2 than does NFAT does. Affinity for the MEF2 can be determined *in vitro* by performing kinetic studies on binding rates.

Other compounds may be developed based on computer modeling and predicted higher order structure, both of the calcineurin, NFAT and of the identified NFAT target molecules. This approach has proved successful in developing inhibitors for a number of receptor-ligand interactions.

4. Genetic Constructs and Gene Transfer

In particular embodiments, it will be desirable to place a calcineurin or an NFAT gene into expression constructs and monitor their effect on the muscle fiber specific gene expression. For example, calcineurin or NFAT may be tested by introducing into cultured muscle fiber cells an expression construct comprising a promoter operably linked to the calcineurin or NFAT gene or genes and monitoring the expression of the genes. A slow muscle fiber specific effect may be demonstrated when there is an increase in expression of slow muscle fiber specific genes.

Expression constructs also are used in generating transgenic animals, such constructs have a promoter for expression of the construct in an animal cell and a region encoding a gene product which modulates transcription of at least one gene that is expressed in myocytes in response to a particular signal responsible for fiber specific expression. In other embodiments, the expression construct encodes an antisense oligo- or polynucleotide is placed in a replicable cloning vehicle that supports expression of the antisense molecule for the therapeutic purposes discussed above.

a. Genetic Constructs

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for gene products in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding genes of interest.

i. Promoters

Transcriptional regulatory elements which are suitable for use in the present invention include which direct the transcription of a coding region to which they are operably linked preferentially in myocytes. By "preferentially" is meant that the expression of the transgene in myocytes is at least about 10-fold, more preferably at least about 10-fold to about 50-fold, even more preferably at least about 50-fold to 100-fold, even more preferably more than 100-fold greater than that in non-myocytes. Preferably, expression of the transgene is below detectable

limits in cells other than myocytes, as indicated by reporter gene assays well known to those of skill in the art.

The nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, β-actin, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. For example in the case where expression of a transgene, or transgenes when a multicistronic vector is utilized, is toxic to the cells in which the vector is produced in, it may be desirable to prohibit or reduce expression of one or more of the transgenes. Examples of transgenes that may be toxic to the producer cell line are pro-apoptotic and cytokine genes. Several inducible promoter systems are available for production of viral vectors where the transgene product may be toxic.

The ecdysone system (Invitrogen, Carlsbad, CA) is one such system. This system is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility. The system is based on the heterodimeric ecdysone receptor of Drosophila, and when ecdysone or an analog such as muristerone A binds to the receptor, the receptor activates a promoter to turn on expression of the downstream transgene high levels of mRNA transcripts are attained. In this system, both monomers of the heterodimeric receptor are constitutively expressed from one vector, whereas the ecdysone-

responsive promoter which drives expression of the gene of interest is on another plasmid. Engineering of this type of system into the gene transfer vector of interest would therefore be useful. Cotransfection of plasmids containing the gene of interest and the receptor monomers in the producer cell line would then allow for the production of the gene transfer vector without expression of a potentially toxic transgene. At the appropriate time, expression of the transgene could be activated with ecdysone or muristeron A.

Another inducible system that would be useful is the Tet-Off™ or Tet-On™ system (Clontech, Palo Alto, CA) originally developed by Gossen and Bujard (Gossen and Bujard, 1992; Gossen *et al.*, 1995). This system also allows high levels of gene expression to be regulated in response to tetracycline or tetracycline derivatives such as doxycycline. In the Tet-On™ system, gene expression is turned on in the presence of doxycycline, whereas in the Tet-Off™ system, gene expression is turned on in the absence of doxycycline. These systems are based on two regulatory elements derived from the tetracycline resistance operon of *E. coli*. The tetracycline operator sequence to which the tetracycline repressor binds, and the tetracycline repressor protein. The gene of interest is cloned into a plasmid behind a promoter that has tetracycline-responsive elements present in it. A second plasmid contains a regulatory element called the tetracycline-controlled transactivator, which is composed, in the Tet-Off™ system, of the VP16 domain from the herpes simplex virus and the wild-type tertracycline repressor. Thus, in the absence of doxycycline, transcription is constitutively on. In the Tet-On™ system, the tetracycline repressor is not wild-type and in the presence of doxycycline activates transcription. For gene transfer vector production, the Tet-Off™ system would be preferable so that the producer cells could be grown in the presence of tetracycline or doxycycline and prevent expression of a potentially toxic transgene, but when the vector is introduced to the patient, the gene expression would be constitutively on.

In some circumstances, it may be desirable to regulate expression of a transgene in a gene transfer vector. For example, different viral promoters with varying strengths of activity may be utilized depending on the level of expression desired. In mammalian cells, the CMV immediate early promoter if often used to provide strong transcriptional activation. Modified versions of the CMV promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. When expression of a transgene in hematopoetic cells is desired, retroviral promoters such as the LTRs from MLV or MMTV are often used. Other

viral promoters that may be used depending on the desired effect include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters such as from the E1A, E2A, or MLP region, AAV LTR, cauliflower mosaic virus, HSV-TK, and avian sarcoma virus.

Similarly tissue specific promoters may be used to effect transcription in specific tissues or cells so as to reduce potential toxicity or undesirable effects to non-targeted tissues. For example, promoters such as the PSA, probasin, prostatic acid phosphatase or prostate-specific glandular kallikrein (hK2) may be used to target gene expression in the prostate. Similarly, the following promoters may be used to target gene expression in other tissues.

It is envisioned that any of the above promoters alone or in combination with another may be useful according to the present invention depending on the action desired. In addition, this list of promoters is should not be construed to be exhaustive or limiting, those of skill in the art will know of other promoters that may be used in conjunction with the promoters and methods disclosed herein.

ii. Enhancers

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells *via* receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and

Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kB of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

iii. Polyadenylation Signals

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human or bovine growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

b. Gene Transfer

There are a number of ways in which expression vectors may introduced into cells. In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. In other embodiments, non-viral delivery is contemplated. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). Delivery mechanisms are discussed in further detail herein below.

i. Non-viral transfer

The present section provides a discussion of methods and compositions of non-viral gene transfer. DNA constructs of the present invention are generally delivered to a cell, and in certain situations, the nucleic acid or the protein to be transferred may be transferred using non-viral methods.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988).

Once the construct has been delivered into the cell the nucleic acid encoding the particular gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In another particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline condensed globules (Radler *et al.*, 1997). These DNA-lipid complexes are potential non-viral vectors for use in gene delivery.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Using the β-lactamase gene, Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection. Also included are various commercial approaches involving "lipofection" technology.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

Other vector delivery systems which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.*, (1987) employed lactosyl-ceramide, a galactose-terminal

asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky *et al.*, (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner *in vivo* and express CAM.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene application refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

ii. Viral Transfer

Adenovirus. One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct

and (b) to express an antisense polynucleotide, a protein, a polynucleotide (*e.g.*, ribozyme, or an mRNA) that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retroviruses, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. As used herein, the term "genotoxicity" refers to permanent inheritable host cell genetic alteration. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification of normal derivatives. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

The E3 region encodes proteins that appears to be necessary for efficient lysis of Ad infected cells as well as preventing TNF-mediated cytolysis and CTL mediated lysis of infected cells. In general, the E4 region encodes is believed to encode seven proteins, some of which

activate the E2 promoter. It has been shown to block host mRNA transport and enhance transport of viral RNA to cytoplasm. Further the E4 product is in part responsible for the decrease in early gene expression seen late in infection. E4 also inhibits E1A and E4 (but not E1B) expression during lytic growth. Some E4 proteins are necessary for efficient DNA replication however the mechanism for this involvement is unknown. E4 is also involved in post-transcriptional events in viral late gene expression; *i.e.*, alternative splicing of the tripartite leader in lytic growth. Nevertheless, E4 functions are not absolutely required for DNA replication but their lack will delay replication. Other functions include negative regulation of viral DNA synthesis, induction of sub-nuclear reorganization normally seen during adenovirus infection, and other functions that are necessary for viral replication, late viral mRNA accumulation, and host cell transcriptional shut off.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993; Shenk, 1978).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey

embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.*, (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking is initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical, medical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.*, (1986), or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression investigations (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene transfer (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993), intranasal inoculation (Ginsberg *et al.*, 1991), aerosol administration to lung (Bellon, 1996) intra-peritoneal administration (Song *et al.*, 1997), Intra-pleural injection (Elshami *et al.*, 1996) administration to the bladder using intra-vesicular administration (Werthman, *et al.*, 1996), Subcutaneous injection including intraperitoneal, intrapleural, intramuscular or subcutaneously) (Ogawa, 1989) ventricular injection into myocardium (heart, French *et al.*, 1994), liver perfusion (hepatic artery or portal vein, Shiraishi *et al.*, 1997) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

Retrovirus. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, *gag*, *pol*, and *env* that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol*, and *env* genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux

et al., 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

Herpesvirus. Because herpes simplex virus (HSV) is neurotropic, it has generated considerable interest in treating nervous system disorders. Moreover, the ability of HSV to establish latent infections in non-dividing neuronal cells without integrating in to the host cell chromosome or otherwise altering the host cell's metabolism, along with the existence of a promoter that is active during latency makes HSV an attractive vector. And though much attention has focused on the neurotropic applications of HSV, this vector also can be exploited for other tissues given its wide host range.

Another factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (temporal, strength, etc.) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations.

HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a

lessened need for repeat dosings. For a review of HSV as a gene transfer vector, see Glorioso *et al.*, (1995).

HSV, designated with subtypes 1 and 2, are enveloped viruses that are among the most common infectious agents encountered by humans, infecting millions of human subjects worldwide. The large, complex, double-stranded DNA genome encodes for dozens of different gene products, some of which derive from spliced transcripts. In addition to virion and envelope structural components, the virus encodes numerous other proteins including a protease, a ribonucleotides reductase, a DNA polymerase, a ssDNA binding protein, a helicase/primase, a DNA dependent ATPase, a dUTPase and others.

HSV genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974; Honess and Roizman 1975; Roizman and Sears, 1995). The expression of α genes, the first set of genes to be expressed after infection, is enhanced by the virion protein number 16, or α-transducing factor (Post *et al.*, 1981; Batterson and Roizman, 1983). The expression of β genes requires functional α gene products, most notably ICP4, which is encoded by the α4 gene (DeLuca *et al.*, 1985). γ genes, a heterogeneous group of genes encoding largely virion structural proteins, require the onset of viral DNA synthesis for optimal expression (Holland *et al.*, 1980).

In line with the complexity of the genome, the life cycle of HSV is quite involved. In addition to the lytic cycle, which results in synthesis of virus particles and, eventually, cell death, the virus has the capability to enter a latent state in which the genome is maintained in neural ganglia until some as of yet undefined signal triggers a recurrence of the lytic cycle. Avirulent variants of HSV have been developed and are readily available for use in gene transfer contexts (U.S. Patent 5,672,344).

Adeno-Associated Virus. Recently, adeno-associated virus (AAV) has emerged as a potential alternative to the more commonly used retroviral and adenoviral vectors. While studies with retroviral and adenoviral mediated gene transfer raise concerns over potential oncogenic properties of the former, and immunogenic problems associated with the latter, AAV has not been associated with any such pathological indications.

In addition, AAV possesses several unique features that make it more desirable than the other vectors. Unlike retroviruses, AAV can infect non-dividing cells; wild-type AAV has been characterized by integration, in a site-specific manner, into chromosome 19 of human cells (Kotin and Berns, 1989; Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991); and AAV also possesses anti-oncogenic properties (Ostrove *et al.*, 1981; Berns and Giraud, 1996). Recombinant AAV genomes are constructed by molecularly cloning DNA sequences of interest between the AAV ITRs, eliminating the entire coding sequences of the wild-type AAV genome. The AAV vectors thus, produced lack any of the coding sequences of wild-type AAV, yet retain the property of stable chromosomal integration and expression of the recombinant genes upon transduction both *in vitro* and *in vivo* (Berns, 1990; Berns and Bohensky, 1987; Bertran *et al.*, 1996; Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997a). Until recently, AAV was believed to infect almost all cell types, and even cross species barriers. However, it now has been determined that AAV infection is receptor-mediated (Ponnazhagan *et al.*, 1996; Mizukami *et al.*, 1996).

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription. The sequence of AAV is provided by Srivastava *et al.*, (1983), and in U.S. Patent 5,252,479 (entire text of which is specifically incorporated herein by reference).

The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-structural proteins apparently are derived from the longer of the transcripts, and three virion proteins all arise from the smallest transcript.

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of

the helpers is adenovirus, and many "early" functions for this virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

Vaccinia Virus. Vaccinia virus vectors have been used extensively because of the ease of their construction, relatively high levels of expression obtained, wide host range and large capacity for carrying DNA. Vaccinia contains a linear, double-stranded DNA genome of about 186 kb that exhibits a marked "A-T" preference. Inverted terminal repeats of about 10.5 kb flank the genome. The majority of essential genes appear to map within the central region, which is most highly conserved among poxviruses. Estimated open reading frames in vaccinia virus number from 150 to 200. Although both strands are coding, extensive overlap of reading frames is not common.

At least 25 kb can be inserted into the vaccinia virus genome (Smith and Moss, 1983). Prototypical vaccinia vectors contain transgenes inserted into the viral thymidine kinase gene *via* homologous recombination. Vectors are selected on the basis of a tk-phenotype. Inclusion of the untranslated leader sequence of encephalomyocarditis virus, the level of expression is higher than that of conventional vectors, with the transgenes accumulating at 10% or more of the infected cell's protein in 24 h (Elroy-Stein *et al.*, 1989).

Lentiviruses. Lentiviruses can also be used as vectors in the present application. In addition to the long-term expression of the transgene provided by all retroviral vectors, lentiviruses present the opportunity to transduce nondividing cells and potentially achieve regulated expression. The development of lentiviral vectors requires the design of transfer vectors to ferry the transgene with efficient encapsidation of the transgene RNA and with full expression capability, and of a packaging vector to provide packaging machinery *in trans* but without helper virus production. For both vectors, a knowledge of packaging signal is required- the signal to be included in the transfer vector but excluded from the packaging vector. Exemplary human lentiviruses are human immunodeficiency virus type 1 and type 2 (HIV-1 and HIV-2). HIV-2 is likely better suited for gene transfer than HIV-1 as it is less pathogenic and thus safer during design and production; its desirable nuclear import and undesirable cell-cycle arrest functions are segregated on two separate genes (Arya *et al.*, 1998; Blomer *et al.*, 1997).

c. Selection Methods

Primary mammalian cell cultures may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

One embodiment of the foregoing involves the use of gene transfer to immortalize cells for the production of proteins. The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. The generation of recombinant expression vectors, and the elements included therein, are discussed above. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell in question.

Examples of useful mammalian host cell lines are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, NIH3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and process the gene product in the manner desired. Such modifications

(*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the foreign protein expressed.

Thus, following introduction of the expression construct into the cells, expression of the reporter gene can be determined by conventional means. Any assay which detects a product of the reporter gene, either by directly detecting the protein encoded by the reporter gene or by detecting an enzymatic product of a reporter gene-encoded enzyme, is suitable for use in the present invention. Assays include colorimetric, fluorimetric, or luminescent assays or even, in the case of protein tags, radioimmunoassays or other immunological assays. Transfection efficiency can be monitored by co-transfecting an expression construct comprising a constitutively active promoter operably linked to a reporter gene.

A number of selection systems may be used including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk-*, *hgprt-* or *aprt-* cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to *gpt*; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

Animal cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

5. Screening For Modulators Of Muscle Fiber Composition

The present invention also contemplates the screening of compounds for their ability to modulate the composition of fibers of a particular muscle. The ability to create cellular, organ and organismal systems which mimmic the effects of a constitutively activated calcineurin on muscle cells provides an ideal setting in which to test various compounds for therapeutic activity. Particularly preferred compounds will be those useful in modulating the calcineurin

levels in the particular muscle and therefore modulating muscle fiber composition. In the screening assays of the present invention, the candidate substance may first be screened for basic biochemical activity -- *e.g.*, binding to a target molecule -- and then tested for its ability to modulate calcineurin activity, at the cellular, tissue or whole animal level.

a. Modulators and Assay Formats

i. Assay Formations

The present invention provides methods of screening for modulators of muscle fiber phenotype. It is contemplated that these screening techniques will prove useful in the identification of compounds that alter the phenotype of a muscle fiber from fast to slow or vice verse. It is contemplated that the modulator will achieve this effect by acting on the calcineurin pathway in the muscle cells

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to alter muscle fiber phenotype, generally including the steps of:

(a) providing a skeletal muscle cell expressing an NFAT and/or a MEF2 gene;
(b) contacting said cell with a candidate modulator; and
(c) monitoring said cell for a phenotype that is absent when said cell is not treated with said candidate modulator.

To identify a candidate substance as being capable of altering the phenotype of the muscle cell in the assay above, one would measure or determine various characteristics of the cell, for example, calcinuerin activity, growth, Ca^{2+}-dependent gene expression and the like in the absence of the added candidate substance. One would then add the candidate substance to the cell and determine the response in the presence of the candidate substance. A candidate substance which increases stimulates or otherwise potentiates calcineurin activity or function is indicative of said modulator being a stimulator of slow fiber formation whereas a modulator which decreases inhibits or otherwise abrogate calcineurin activity or function is indicative of said modulator being a stimulator of fast fiber formation. In the screening assays of the present invention, the compound is added to the cells, over period of time and in various dosages, and the selected phenotype is measured.

In particularly preferred aspects, the cells express an NFAT and/or a MEF2 gene. In certain embodiments, the other genes involved in the NFAT pathway may be altered to achieve the same effect, such as a mutant form of MEF2 that is capable of functioning without the assistance of NFAT. It also is contemplated that the cells may express a fiber-specific gene having a promoter containing MEF2 and NFAT binding sites.

ii. Inhibitors and Activators of Calcineurin

An activator or stimulator of calcineurin activity according to the present invention may be one which exerts its activatory effect upstream or downstream of calcineurin, or on calcineurin directly. Regardless of the type of activator identified by the present screening methods, the effect of such a compound results in activation of genes that are responsible for slow twitch fiber phenotype and therefore leads to an increase in the presence of slow fiber phenotype over that of a fast fiber phenotype. In certain embodiments, a downstream signaling element may be installed into the cell such that an increase in a signal would indicate an increase in activity in the pathway. One conceivable signal would be a gene such as green fluorescent protein linked to a regulatory control region that was activated by NFAT/MEF2.

In alternative embodiments, the present invention provides methods for identifying an inhibitor of the calcineurin pathway in muscle fibers thereby leading to an increase in the proportion of fast fiber in the muscle composition. Such an inhibitor will serve to prevent the effect of calcineurin being mediated through an interaction between NFAT and MEF2 (or other transcription factors that bind to a dephosphorylated or activated NFAT protein). Thus, an inhibitor as described in this embodiment may be one which inhibits the dephosphorylation of NFAT, inhibits the expression of NFAT or one which prevents the interaction of NFAT and MEF2.

iii. Candidate Substances

As used herein the term "candidate substance" refers to any molecule that may potentially modulate calcineurin activity. The candidate substance may be a protein or fragment thereof, a small molecule, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to other known modulators of calcineurin activity. For example, known activators of calcineurin include Ca2+ and calmodulin whereas known inhibitors include cyclosporin A

and FK506. Such an endeavor often is know as "rational drug design," and includes not only comparisons with know inhibitors, but predictions relating to the structure of target molecules.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or target compounds. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for an NFAT molecule, or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches.

It also is possible to use antibodies to ascertain the structure of a target compound or inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotype would be expected to be an analog of the original antigen. The anti-idiotype could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

On the other hand, one may simply acquire, from various commercial sources, small molecule libraries that are believed to meet the basic criteria for useful drugs in an effort to "brute force" the identification of useful compounds. Screening of such libraries, including combinatorially generated libraries (*e.g.*, peptide libraries), is a rapid and efficient way to screen large number of related (and unrelated) compounds for activity. Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third and fourth generation compounds modeled of active, but otherwise undesirable compounds.

Candidate compounds may include fragments or parts of naturally-occurring compounds or may be found as active combinations of known compounds which are otherwise

inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be polypeptide, polynucleotide, small molecule or any other compounds that may be designed through rational drug design starting from known modulators of calcineurin.

"Effective amounts" in certain circumstances are those amounts effective to reproducibly increase or decrease the calcineurin activity from the cell in comparison to their normal levels. Compounds that achieve significant appropriate changes in muscle cell phenotype will be used.

Significant changes in muscle cell phenotype, *e.g.*, as measured using myocyte growth, Ca^{2+} response, muscle fiber specific gene expression, and the like are represented by a changes in activity of at least about 30%-40%, and most preferably, by changes of at least about 50%, with higher values of course being possible. The active compounds of the present invention also may be used for the generation of antibodies which may then be used in analytical and preparatory techniques for detecting and quantifying further such modulators.

It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

b. *In vitro* Assays

A quick, inexpensive and easy assay to run is a binding assay. Binding of a molecule to a target may, in and of itself, be alter the activity of calcineurin, due to steric, allosteric or charge-charge interactions. This can be performed in solution or on a solid phase and can be utilized as a first round screen to rapidly eliminate certain compounds before moving into more sophisticated screening assays. In one embodiment of this kind, the screening of compounds that bind to the calcineurin molecule or fragment thereof is provided

The target may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the target or the compound may be labeled, thereby permitting determining of binding. In another embodiment, the assay may measure the inhibition of binding of a target to a natural or artificial substrate or binding partner (such as NFAT and MEF2). Competitive binding assays can be performed in which one of the agents (NFAT3 for example) is labeled. Usually, the target will be the labeled species, decreasing the chance that the labeling will interfere with the binding moiety's function. One may measure the amount of free label versus bound label to determine binding or inhibition of binding.

A technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with, for example, NFAT and washed. Bound polypeptide is detected by various methods.

Purified target, such as NFAT, can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the polypeptide can be used to immobilize the polypeptide to a solid phase. Also, fusion proteins containing a reactive region (preferably a terminal region) may be used to link an active region (*e.g.*, the C-terminus of NFAT) to a solid phase.

c. ***In cyto* Assays**

Various cell lines that exhibit characteristics of fast or slow muscle fiber type can be utilized for screening of candidate substances. Such cells can be used in assays in which the compound would be formulated appropriately, given its biochemical nature, and contacted with a target cell.

Depending on the assay, culture may be required. As discussed above, the cell may then be examined by virtue of a number of different physiologic assays (growth, size, Ca^{++} effects, fiber specific gene expression). Alternatively, molecular analysis may be performed in which the function of calcineurin and related pathways may be explored. This involves assays such as those for protein expression, enzyme function, substrate utilization, mRNA expression (including differential display of whole cell or polyA RNA) and others.

d. *In vivo* Assays

The present invention particularly contemplates the use of various animal models. Here, transgenic mice expressing a constitutively activated calcineurin can be used to monitor the effects of the candidate substance in a whole animal system. The generation of these animals has been described elsewhere in this document. These models can, therefore be used not only screen for modulators of muscle specific fiber.

Treatment of these animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route the could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated are systemic intravenous injection, regional administration via blood or lymph supply.

Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Such criteria include, but are not limited to, survival, alteration in muscle size or mass, and improvement of general physical state including activity. It also is possible to perform histologic studies on tissues from these mice, or to examine the molecular state of the cells, which includes cell size or alteration in the expression of muscle fiber specific genes. U.S. Patent 5,628,328 provides methods for determining muscle mass in a human subject useful for monitoring athletic conditioning, weight loss programs, nutritional deficiencies, and disease states which cause muscle wasting.

6. **Pharmaceutical Compositions**

Where clinical application of an active ingredient (drugs, polypeptides, antibodies or liposomes containing antisense oligo- or polynucleotides or expression vectors) is undertaken, it will be necessary to prepare a pharmaceutical composition appropriate for the intended application. Generally, this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate buffers to render the complex stable and allow for uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the active ingredient, as discussed above, further dispersed in pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of therapeutic compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact

concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, a controlled release patch, salve or spray.

The therapeutic compositions of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. A preferred embodiment delivery route, for the treatment of a disseminated disease state is systemic, however, regional delivery is also contemplated.

An effective amount of the therapeutic composition is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection desired.

Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the patient, the route of administration, the intended goal of treatment and the potency, stability and toxicity of the particular therapeutic substance.

7. Transgenic Animals/Knockout Animals

In one embodiment of the invention, transgenic animals are produced which contain a functional transgene encoding a functional calcineurin polypeptide or variants thereof. Transgenic animals expressing calcineurin transgenes, recombinant cell lines derived from such animals and transgenic embryos may be useful in methods for screening for and identifying agents that induce or repress function of calcineurin. Alternative transgenic animals that may be employed herein include those which have a functional NFAT transgene. Transgenic animals of the present invention also can be used as models for studying indications such as muscular dystrophy.

In one embodiment of the invention, a calcineurin transgene is introduced into a non-human host to produce a transgenic animal expressing a human or murine calcineurin gene. The transgenic animal is produced by the integration of the transgene into the genome in a manner that permits the expression of the transgene. Methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Patent 4,873,191; which is incorporated herein by reference), Brinster *et al.*, 1985; which is incorporated herein by reference in its entirety) and in "Manipulating the Mouse Embryo; A Laboratory Manual" 2nd edition (eds., Hogan, Beddington, Costantimi and Long, Cold Spring Harbor Laboratory Press, 1994; which is incorporated herein by reference in its entirety).

It may be desirable to replace the endogenous calcineurin by homologous recombination between the transgene and the endogenous gene; or the endogenous gene may be eliminated by deletion as in the preparation of "knock-out" animals. Typically, a specific gene flanked by genomic sequences is transferred by microinjection into a fertilized egg. The microinjected eggs are implanted into a host female, and the progeny are screened for the expression of the transgene. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, mammals, and fish. Within a particularly preferred embodiment, transgenic mice are generated which overexpress calcineurin or express a mutant form of the polypeptide. Alternatively, the absence of a calcineurin in "knock-out" mice permits the study of the effects that loss of calcineurin protein has on a cell *in vivo*. Knock-out mice also provide a model for the development of calcineurin-related defects in muscle development.

As noted above, transgenic animals and cell lines derived from such animals may find use in certain testing experiments. In this regard, transgenic animals and cell lines capable of expressing wild-type or mutant calcineurin may be exposed to test substances. These test substances can be screened for the ability to enhance wild-type calcineurin expression and or function or impair the expression or function of mutant calcineurin.

8. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus, can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Materials and Methods

Cell culture and transfection conditions

NIH 3T3 cells or C2C12 myogenic cells were cultured, transfected with plasmid vectors, and assayed for luciferase and ß-galactosidase, as previously described (Grayson *et al.*, 1995; Grayson *et al.*, 1998). Each 35 mm dish of cells was cotransfected with promoter-reporter plasmid (0.5 µg), an expression plasmid that uses the CMV promoter to force expression of a constitutively active form of calcineurin (Manalan and Klee, 1983; O'Keefe *et al.*, 1992), or empty vector (pCI-NEO; 0.5 µg), along with a CMV-lacZ plasmid (0.5 µg) as an internal control for transfection efficiency. For dose-response experiments (FIG. 2), the total input DNA and the amount of promoter-reporter plasmid and CMV-lacZ was held constant, but the ratio of calcineurin expression vector to empty vector was varied.

Plasmid constructions

The expression plasmid used to stimulate calcineurin-regulated gene transcription was constructed by linking a CMV promoter carried in pCI-NEO (Promega) to a truncated variant of calcineurin A from which the carboxyl terminal region containing the autoinhibitory domain and a portion of the calmodulin binding domain was deleted (O'Keefe *et al.*, 1992). This form

of calcineurin exhibits constitutive phosphatase activity, and is not subject to regulation by calcium-calmodulin in the manner of the native protein (O'Keefe *et al.*,-1992). Promoter-reporter constructs were designed by linking the luciferase gene carried in pGL3 (Promega) to upstream promoter regions from the myoglobin (Mb), troponin I slow (TnIs), and muscle creatine kinase (MCK) genes, each of which have been shown previously to recapitulate the expression pattern of the respective endogenous genes when linked to a reporter gene and introduced into transgenic mice (Parsons *et al.*, 1993; Levitt *et al.*, 1995; Shield *et al.*, 1996).

Other promoter-reporter plasmids used as controls (CMV-luciferase; TATA-luciferase; CMV-lacZ), or to identify upstream regulatory elements involved in transducing the signal derived from activated calcineurin (Mb380; MbΔA/T; MbΔCCAC; CCAC-TATA; A/T-TATA; CCAC-A/T-TATA) have been described in previous publications from this laboratory (Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995; Grayson *et al.*, 1998). Reporter constructions bearing five copies of the upstream NFAT response element from the myoglobin promoter (NRE-TATA and NRE-CCAC-A/T-TATA) were based on the oligonucleotide sequence 5'-AACCAGGAAATAGGATGCCCT-3' (SEQ ID NO:1), and its complementary strand, representing nucleotide positions -694 to -674 in the human myoglobin promoter (underlined bases illustrate the NFAT consensus binding motif).

Putative NFAT binding sites within the myoglobin and troponin I slow promoters were disrupted using a PCR-based mutagenesis procedure, as described (Yang *et al.*, 1997). The specific nucleotide sequence modifications included: myoglobin promoter (-690) AGGAAATA to GTCGACTA and (-232, reverse strand) TGGAAAGA to CTCGAGGA; TnI slow promoter (-738) AGGAAAC to AGCTAGC and (-639) TGGAAACA to ACTAGTCA.

Plasmids used to express NFAT-GFP fusion proteins were constructed in pEGFP-N1 (Clontech), using cDNA sequences encoding full length (amino acids 1-716) or truncated (amino acids 319-716) NFATc (Northrop *et al.*, 1994), modified at the carboxyl terminus for fusion in the correct reading frame to GFP. In the construct designed to express the truncated NFATc-GFP fusion protein (ΔNFATc-GFP), the native leucine residue at position 319 was converted to a methionine initiation codon. In both NFATc-GFP and ΔNFATc-GFP, the native stop codon was replaced with a 7 amino acid insertion preceeding the GFP coding sequence.

Fluorescence microscopy

An Olympus IMT-2 inverted fluorescence photomicroscope with FITC illumination and detection was used for evaluation and photography of C2C12 cells transfected with GFP expression plasmids. GFP fluorescence (excitation peak = 488nm, emission peak = 507nm) was photographed with Kodak Elite II 400 ASA slide film using an Olympus SC35 SLR camera back.

Histochemical analysis of fiber type in muscles from intact animals

Adult rats were treated with cyclosporin A (5 mg/kg) or vehicle administered by intraperitoneal injection daily for 6 weeks. Animal care was in accordance with institutional guidelines. Sections of soleus muscles from 7 animals in each group were histochemically stained for myosin ATPase activity at pH 4.54, as described (Brooke and Kaiser, 1970). The proportion of fast and slow fibers was quantified by three observers who were blinded to the treatment status of the animals. Fibers expressing fast myosin were identified in 8μm cryosections sections of the same muscles, post-fixed in 4% paraformaldehype, by immunohistochemical analysis using a commercially available mouse monoclonal antibody (MY-32: Sigma, St. Louis, MO; 1:400) and LRSC goat anti-mouse IgG (Jackson Immunochemicals, West Grove, PA; 1:50). Nuclei were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR) at 0.6μg/ml for 10 minutes.

EXAMPLE 2

Selective Activation of Slow Fiber-Specific Promoters By Forced Expression Of a Constitutively Active Form Of Calcineurin

The myoglobin and troponin I slow (TnIs) genes are expressed selectively in slow, oxidative skeletal muscle fibers (Levitt *et al.*, 1995; Garry *et al.*, 1996), while the muscle creatine kinase (MCK) gene is expressed most abundantly in the fast, glycolytic myofiber subtype (Yamashita and Yoshioka 1991). To test whether these genes might respond differently to a calcineurin-stimulated signaling pathway, skeletal myogenic cells were transfected with reporter genes linked to well-characterized control regions from these genes, along with an expression vector encoding a constitutively active (calcium-insensitive) form of calcineurin that retains sensitivity to inhibition by cyclosporin A (Manalan and Klee, 1983; O'Keefe *et al.*, 1992).

Transcriptional activity of the slow fiber-specific myoglobin and TnIs promoters was stimulated in cultured skeletal myotubes (C2C12) by active calcineurin, as measured by expression of luciferase in cotransfection assays (FIG. 1). In contrast, activity of the fast-fiber specific MCK promoter, or of other strong (CMV) or weak (minimal TATA element) promoters, was unaffected by activated calcineurin. The induction of the myoglobin promoter in the presence of the calcineurin expression plasmid was inhibited by cyclosporin A. This result indicates the specificity of the response, since the effect of cyclosporin A is to bind cyclophilin and form a protein complex that binds calcineurin and inhibits its protein phosphatase activity (Liu *et al.*, 1991). The same relative potency of calcineurin-dependent transactivation (myoglobin and TnIs >> MCK, CMV, or TATA) was observed in Sol8 myotubes. a different myogenic cell line. In contrast, forced expression of activated calcineurin had no effect on promoter activity in undifferentiated myoblasts or in 3T3 fibroblasts (FIG. 1), demonstrating a requirement for muscle-specific factors in the calcineurin-stimulated pathway for transcriptional control of the myoglobin and TnIs promoters.

EXAMPLE 3

Calcineurin-stimulated trans-activation of slow fiber-specific promoters requires nucleotide sequence motifs characteristic of NFAT binding sites

The finding that the myoglobin and TnIs promoters can be transcriptionally regulated by a calcineurin-dependent mechanism suggested the participation of NFAT transcription factors in the signaling cascade. Examination of the complete nucleotide sequences of these functionally defined transcriptional control regions (2.0 and 4.2 kb, respectively) revealed two 8 bp elements within each that match the consensus binding sequence for NFAT transcription factors (Rao *et al.*, 1997). The response to activated calcineurin of the native promoter sequences was compared to that of mutated promoters in which these putative NFAT recognition elements were ablated by site-directed mutatagenesis.

Disruption of putative NFAT recognition elements within both the myoglobin (FIG. 2A) and TnIs (FIG. 2B) promoters diminished the response to activated calcineurin, indicating that the transactivation mechanism is likely to involve DNA binding of NFAT proteins. Transduction of the calcineurin-directed signal to the native myoglobin and TnIs promoters exhibited a saturable dose-response relationship with respect to the activated calcineurin

expression plasmid, and diminished reporter gene activation was evident across the entire dose range examined. Some degree of calcineurin-dependent transactivation persisted after ablation of identifiable NFAT binding sites within these transcriptional control regions. Thus, either cryptic binding sites for NFAT proteins that cannot be recognized by inspection of the DNA sequence are present, or calcineurin-dependent signaling to these promoters can be driven without direct DNA binding of NFAT proteins.

Nuclear localization of NFAT proteins in skeletal myocytes is under the control of calcineurin (FIG. 2C), as predicted from previously published results in lymphocytes (Timmerman *et al.*, 1996). A fusion protein linking Green Fluorescent Protein (GFP) to full length NFATc (NFATc-GFP) is excluded from the nucleus in C2C12 cells under basal conditions, but undergoes nuclear translocation in the presence of activated calcineurin. As controls, an NFATc-GFP fusion protein lacking amino acids 1-318 of NFATc (ΔNFATc-GFP) is constitutively localized to the nucleus in the absence of activated calcineurin, while native GFP is distributed across both cytoplasmic and nuclear compartments. The amino terminal segment of NFAT proteins (missing in ΔNFATc-GFP) includes the conserved SPRIEIT motif that constitutes the calcineurin targeting site (Arambaru *et al.*, 1998).

EXAMPLE 4

Calcineurin-stimulated trans-activation of slow fiber-specific promoters requires collaboration among multiple transcription factors

Muscle-specific transcription factors are required for calcineurin-dependent activation of the myoglobin and TnI slow promoters, since no response was observed in a fibroblast cell background (FIG. 1). Previously, two conserved upstream response elements have been defined within the myoglobin promoter, both of which are required for transcriptional activity in skeletal myotubes or cardiac myocytes (Devlin *et al.*, 1989; Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995; Grayson *et al.*, 1998). These CCAC and A/T elements represent binding sites for Sp1 and MEF2 proteins, respectively, and function synergistically in muscle-specific gene regulation (Grayson *et al.*, 1995; Grayson *et al.*, 1998). This prior work established a molecular basis for muscle-specific expression of myoglobin, but failed to account for selective expression of myoglobin in slow fiber types, since Sp1 and MEF2 proteins are equally abundant in slow and fast fibers.

A myoglobin promoter segment truncated to nucleotides -373 to +7 (Mb380) was sufficient for muscle-specific expression in prior experiments (Devlin *et al.*, 1989; Bassel-Duby *et al.*, 1993; Grayson *et al.*, 1995) and was responsive to calcineurin stimulation in the current studies (FIG. 3A). This region includes the CCAC and A/T motifs required for muscle-specific promoter activity, as well as a putative NFAT response element. Nucleotide substitutions within either the CCAC or A/T elements of Mb380 reduced basal transcription in differentiated myotubes, as observed previously, and abrogated the response to calcineurin (FIG. 3A). Thus, mutations that compromise binding of MEF2, Sp1, or other factors to the CCAC and A/T elements interdict the calcineurin-stimulated response, even when the NFAT consensus binding motif at -232 remains intact.

Functional interactions between transcription factors binding to motifs within the myoglobin promoter were examined further by linkage of various combinations of multimerized oligonucleotide cassettes representing cognate binding sites for MEF2, Sp1 and NFAT in promoter-reporter constructions. As assessed by cotransfection assays in C2C12 myotubes (FIG. 3B), forced expression of activated calcineurin only marginally enhanced transcription (< 2-fold) of a construct bearing multiple copies of the CCAC motif. The response to calcineurin was somewhat more robust (3-fold) if multimers of the A/T element were included within the synthetic promoters, either in the absence of heterologous protein binding sites, or when combined with multimerized CCAC sites. A reporter construction bearing multiple copies of the upstream (-690) NFAT response element (NRE) from the myoglobin promoter was minimally stimulated by activated calcineurin (< 2-fold) in this cell background, but a construct combining NRE, A/T and CCAC motifs was potently transactivated (6-fold). These results demonstrate that collaborative interactions among proteins binding to NRE, A/T and CCAC elements from the myoglobin promoter are necessary for optimal transduction of the calcineurin-stimulated signal.

EXAMPLE 5

Administration of the calcineurin antagonist cyclosporin A to intact animals promotes slow-to-fast fiber transformation

To determine whether calcineurin-dependent activation of slow fiber-specific promoters observed in cultured myotubes is pertinent to mature myofibers of intact animals, the proportion of fast versus slow fibers was assessed within soleus muscles of rats treated with

cyclosporin A, a specific inhibitor of calcineurin (Liu *et al.*, 1991; Clipstone and Crabtree 1992). The intraperitoneal administration of an immunosuppressant dose (5 mg/kg/day) of cyclosporin A for 6 weeks uniformly increased the proportion of fast fibers defined either by histochemical staining of myosin ATPase activity (Brooke and Kaiser, 1970), or by specific immunohistochemical staining of fast skeletal myosin. In soleus muscles of 7 control animals, fast (Type II) fibers represented 4-24% (mean 14 $\pm$ 3%) of the total cell population, while 28-37% (mean 31 $\pm$ 1%) of soleus fibers expressed fast myosin in 7 cyclosporin A-treated rats ($p<.001$) (FIG. 4). This result is consistent with the hypothesis that physiological signals acting to establish and maintain the slow, oxidative myofiber phenotype in intact animals are transduced by a calcineurin-dependent pathway. Interdiction of the calcineurin-signaling pathway with cyclosporin A has reciprocal effects on expression of fast and slow myosin isoforms: not only is slow myosin expression reduced but fast myosin expression is enhanced. Thus, calcineurin-dependent signaling both activates slow fiber-specific genes and represses the fast fiber-specific program.

* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Aramburu *et al.*, *Molec. Cell.*, 1:627-637, 1998.

Arya *et al.*, *Hum Gene Ther.*, 9(9):1371-1380, 1998.

Baichwal and Sugden, In: *Gene Transfer*, Kucherlapati R, ed., New York, Plenum Press, 117-148, 1986.

Bassel-Duby, *et al.*, *Circ. Res.*, 73:360-366, 1993.

Batterson and Roizman, *J. Virol.*, 46:371-377, 1983.

Bedzyk *et al.*, *J. Biol. Chem.*, 265:18615, 1990.

Bellon *et al.*, *de Ses Filiales*, 190(1):109-142, 1996.

Benvenisty and Neshif, *Proc. Nat'l. Acad. Sci. USA*, 83:9551-9555, 1986.

Berns and Bohenzky, *Adv. Virus Res.*, 32:243-307, 1987.

Berns and Giraud, *Curr. Top. Microbiol. Immunol.*, 218:1-23, 1996.

Berns, *Microbiol Rev.*, 54:316-329, 1990.

Bertran *et al.*, *J. Virol.*, 70(10):6759-6766, 1996.

Black, B.L. and E.N. Olson, *Ann. Rev. Cell Dev. Biol.*, 14:167-196, 1998.

Blomer *et al.*, *J. Virol.*, 71(9):6641-6649, 1997.

Booth and Baldwin. In *The Handbook of Physiology: Integration of Motor, Circulatory, Respiratory and Metabolic Control During Exercise* (ed. L.B. Rowell and J.T.Shepard), pp.1075-1123. Bethesda:American Physiology Society, 1996.

Borensztajn *et al.*, *Am. J. Physiol.*, 229(2):394-397, 1975.

Briggs and Schachat, *Am. J. Physiol.*, 270(1 Pt 1):C298-C305, 1996.

Brinster *et al.*, *Proc. Nat'l Acad. Sci. USA*, 82:4438-4442, 1985.

Brooke and Kaiser, *Arch. Neurol.*, 23:369-379, 1970.

Caiozzo, *et al.*, *J. Appl. Physiol.*, 76:1764-1773, 1994.

Cameron *et al.*, *Cell*, 83:463-472, 1995.

Chaudhary *et al.*, *Proc. Nat'l. Acad. Sci.*, 87:9491, 1990.

Chen and Okayama, *Mol. Cell Biol.*, 7:2745-2752, 1987.

Chin and Allen, *Physiologist*, 39:A-75, 1996.

Clipstone and Crabtree, *Nature*, 357:695-697, 1992.

Coffin, *In:* Fields BN, Knipe DM, ed., VIROLOGY. New York: Raven Press, pp. 1437-1500, 1990.

Couch *et al.*, *Am. Rev. Resp. Dis.*, 88:394-403, 1963.

Delp *et al.*, *J. Appl. Physiol.*, 83(4):1291-1299, 1997.

DeLuca *et al.*, *J. Virol.*, 56:558-570, 1985.

Devlin *et al.*, *J. Biol. Chem.*, 264:13896-13900, 1989.

DiMario *et al.*, *Nature*, 362:165-167, 1993.

Dolmetsch *et al.*,. *Nature*, 386:855-858, 1997.

Dubensky *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 81:7529-7533, 1984.

Elroy-Stein *et al.*, *Proc. Nat'l Acad. Sci. USA*, 1989.

Elshami *et al.*, *Gene Therapy*, 7(2):141-148, 1996.

Emmel *et al.*, *Science*, 246:1617-1620, 1989.

Fechheimer *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 84:8463-8467, 1987.

Ferkol *et al.*, *FASEB J.*, 7:1081-1091, 1993.

Fraley *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 76:3348-3352, 1979.

French *et al.*, *Circulation*, 90(5):2414-2424, 1994.

Freshner, In *Animal Cell Culture: a Practical Approach* Second Edition, Oxford/New York, IRL Press, Oxford University Press, 1992.

Garry *et al.*,. *Dev. Genet.*, 19: 146-156, 1996.

Ghosh-Choudhury *et al.*, *EMBO J.*, 6:1733-1739, 1987.

Ghosh and Bachhawat, (Wu G, Wu C ed.), New York: Marcel Dekker, pp. 87-104, 1991.

Ginsberg *et al.*, *Proc. Nat'l Acad. of Sci. USA*, 88(5)1651-1655, 1991.

Glorioso *et al.*, *Ann. Rev. Microbiol.*, 49:675-710, 1995.

Gomez-Foix *et al.*, *J. Biol. Chem.*, 267:25129-25134, 1992.

Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.

Graham and Prevec, *Biotechnol.*, 20:363-390, 1992.

Graham and Prevec, *In:* E.J. Murray (ed.), Methods in Molecular Biology: Gene Transfer and Expression Protocol, Clifton, NJ: Humana Press, 7:109-128, 1991.

Graham and Van Der Eb, *Virology*, 52:456-467, 1973.

Graham *et al.*, *J. Gen. Virol.*, 36:59-72, 1977.

Grayson *et al.*, *Mol. Cell. Biol.*, 15: 1870-1878, 1995.

Grayson *et al.*,. *J. Cell. Biochem.*, 1998.

Grunhaus and Horwitz, *Seminar in Virology*, 3:237-252, 1992.

Harland and Weintraub, *J. Cell Biol.*, 101:1094-1099, 1985.

Hennig, and Lomo, *Nature*, 314: 164-166, 1985.

Hersdorffer *et al.*, *DNA Cell Biol.*, 9:713-723, 1990.

Herz and Gerard, *Proc. Nat'l. Acad. Sci. USA*, 90:2812-2816, 1993.

Hoey *et al.*, *Immunity*, 2: 461-472, 1995.

Hogan *et al.*, "Manipulating the Mouse Embryo A Laboratory Manual" 2nd edition eds., Hogan, Beddington, Costantimi and Long, Cold Spring Harbor Laboratory Press, 1994.

Hoh, *Curr. Opin. Rheumatol.*, 4(6):801-808,1992.

Holland *et al.*, *Virology*, 101:10-18, 1980.

Holloszy and Coyle, *J. Appl. Physiol.*, 56: 831-838, 1984.

Honess and Roizman, *J. Virol.*, 14:8-19, 1974.

Honess and Roizman, *J. Virol.*, 16:1308-1326, 1975.

Howlett and Willis WT, *Biochim. Biophys. Acta*, 1363(3):224-230, 1998

Hughes *et al.*, *Mech. Dev.*, 61: 151-163, 1997

Ianuzzo *et al.*, *J. Appl. Physiol.*, 70: 2328-2830.

Ivy JL, *Sports Med.*, 24(5):321-336, 1997

Izumo, S. and V. Mahdavi, *Nature*, 334: 539-542, 1988.

Johnson *et al.*, *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, eds., Chapman and Hall, New York, 1993.

Jones and Shenk, *Cell*, 13:181-188, 1978.

Jostarndt *et al.*, *J. Histochem. Cytochem.*, 44(10):1141-1152, 1996

Kaciuba-Uscilko *et al.*, *Am J Physiol*, 238(6):E518-E523, 1980.

Kaneda *et al.*, *Science*, 243:375-378, 1989.

Karlsson *et al.*, *EMBO J.*, 5:2377-2385, 1986.

Kato *et al.*, *J. Biol. Chem.*, 266:3361-3364, 1991.

Kearns *et al.*, *Gene Ther.*, 3:748-755, 1996.

Klein *et al.*, *Nature*, 327:70-73, 1987.

Kong *et al.*, *J. Biol Chem.*, 269(17): 12963-12967, 1994.

Kong *et al.*, *J. Biol. Chem.*, 269: 12963-12967, 1994.

Kotin and Berns, *Virol.*, 170:460-467, 1989.

Kotin *et al.*, *Genomics*, 10:831-834, 1991.

Kotin *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 87:2211-2215, 1990.

Kudoh *et al., Circ. Res.*, 80:139-146, 1997.

Lam *et al.,. J. Biol. Chem.*, 270: 26511-26522, 1995.

Le Gal La Salle *et al., Science*, 259:988-990, 1993.

Levitt *et al., DNA Cell Biol.*, 14: 599-607, 1995.

Levitt *et al., DNA Cell Biol.* 14(7):599-607, 1995.

Levrero *et al., Gene*, 101:195-202, 1991.

Li *et al.,. Cell*, 71: 1181-1194, 1992.

Liu *et al., Cell*, 66: 807-815, 1991.

Mackie *et al., J. Appl. Physiol.*, 49(5):851-855, 1980.

Mair *et al., J. Appl. Physiol.*, 72(2):656-663, 1992.

Manalan, A.S. and C.B. Klee, *Proc. Nat'l. Acad. Sci., U S A* 80: 4291-4295, 1983.

Mann *et al., Cell*, 33:153-159, 1983.

Marette *et al., Am. J. Physiol.*, 263(2 Pt 1):C443-C452, 1992.

Markowitz *et al., J. Virol.*, 62:1120-1124, 1988.

Massie *et al., Circulation*, 78: 320-326, 1988.

Matsumoto *et al., J. Biotech. Histochem.*, 72(4):191-197, 1997.

McCaffery *et al., Science*, 262:750-754, 1993.

Mercier *et al., Amer. J. Resp. Crit. Care Med.*, 151: 1532-1536, 1995.

Michel *et al., J. Clin. Invest.*, 94: 277-285, 1994.

Mizukami *et al., Virology*, 217:124-130, 1996.

Molkentin *et al., Cell*, 93: 1-20, 1998.

Molkentin, J.D., and E.N. Olson, *Proc. Nat'l. Acad. Sci. USA*, 93, 9366-9373, 1996

Mulligan, *Science*, 260:926-932, 1993.

Myers, EPO 0273085

Nakano *et al., Brain Res. Bull.*, 43(2):149-154, 1997.

Nakayama *et al., Mol. Cell. Biol.*, 16(5):2408-2417, 1996.

Nakayama *et al., Mol. Cell. Biol.*, 16: 2408-2417 1996.

Neufer *et al., Am. J. Physiol.*, 271: C1828-1837, 1996.

Nicolas and Rubenstein, *In: Vectors: A survey of molecular cloning vectors and their uses.* Rodriguez and Denhardt (eds.), Stoneham: Butterworth, pp. 494-513, 1988.

Nicolau and Sene, *Biochem. Biophys. Acta*, 721:185-190, 1982.

Nicolau *et al., Methods Enzymol.*, 149:157-176, 1987.

Nishida *et al., J. Exp. Zool.*, 1:277(4):283-292, 1997.

Nolan, *Cell*, 77, 1-20, 1994.

Northrop *et al.*, *Nature*, 369: 497-502, 1994.

O'Keefe *et al.*, *Nature*, 357: 692-694, 1992.

Ogata and Yamasaki, *Anat. Rec.*, 248(2):214-223, 1997.

Ogawa *et al.*, *J. Mol. Med.*, 73:457-463, 1995.

Olson *et al.*, *Dev. Biol.*, 172: 2-14.

Ontell *et al.*, *Dev. Dynm.*, 198: 203-213, 1993.

Ostrove *et al.*, *Virology*, 113:532-533, 1981.

Parsons *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 90:1726-1730, 1993.

Paskind *et al.*, *Virology*, 67:242-248, 1975.

Perales *et al.*, *Proc. Nat'l. Acad. Sci.*, 91:4086-4090, 1994.

Pette and Vrbova, *Rev. Physiol. Biochem. Pharmacol*, 120:115-202, 1992.

Ponnazhagan *et al.*, *Hum. Gene Ther.*, 8:275-284, 1997a.

Ponnazhagan *et al.*, *J. Gen. Virol.*, 77:1111-1122, 1996.

Post *et al.*, *Cell*, 24:555-565, 1981.

Potter *et al.*, *Proc. Nat'l Acad. Sci. USA*, 81:7161-7165, 1984.

Qin *et al.*, *J. Biol. Chem.*, 272: 25210-25216, 1997.

Racher *et al.*, *Biotechnology Techniques*, 9:169-174, 1995.

Radler *et al.*, *Science*, 275:810-814, 1997.

Ragot *et al.*, *Nature*, 361:647-650, 1993.

Rao *et al.*, *Annu. Rev. Immunol.*, 15:707-747, 1997.

Rao *et al.*, *Mol. Cell. Biol.*, 16(7):3909-3922, 1996.

Renan, *Radiother. Oncol.*, 19:197-218, 1990.

Rich *et al.*, *Hum. Gene Ther.*, 4:461-476, 1993.

Ridgeway, *In:* Rodriguez RL, Denhardt DT, ed. Vectors: A survey of molecular cloning vectors and their uses. Stoneham: Butterworth, pp. 467-492, 1988.

Rippe *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.

Roizman and Sears, In *Fields' Virology*, 3rd Edition, eds. Fields *et al.*, (Raven Press, New York, N.Y.), pp. 2231-2295, 1995.

Rooney *et al.*, *EMBO J.*, 13:625-633, 1994.

Rosenfeld *et al.*, *Cell*, 68:143-155, 1992.

Rosenfeld *et al.*, *Science*, 252:431-434, 1991.

Roux *et al.*, *Proc. Nat'l Acad. Sci. USA*, 86:9079-9083, 1989.

Sabbah *et al.*, *Circulation*, 87:1729-1737, 1993.

Sadoshima and Izumo, *Circ. Res.*, 73:424-438, 1993b.

Sadoshima *et al.*, *Cell*, 75:977-984, 1993a.

Saltin and Gollnick In *Handbook of Physiology: Skeletal Muscle* (ed. L.D.D. Peachey), pp. 555-632. American Physiological Society, Bethesda. 1983.

Samulski *et al.*, *EMBO J.*, 10:3941-3950, 1991.

Schiaffino, S. and C. Reggiani, *Physiol. Rev.* 76:371-423, 1996.

Shen *et al.*, *Magn. Reson, Med.*, 36(1):30-38, 1996.

Shield *et al.*, *Mol. Cell. Biol.*, 16:5058-5068, 1996.

Shiraishi *et al.*, *Transplant Internationl*, 1-0(3):202-206, 1997.

Smith and Moss, *Gene*, 25:21-28, 1983.

Song *et al.*, *Science*, 275:536-540, 1997.

Sreter *et al.*, *Am. J. Physiol.*, 253:C296-C300, 1987.

Srivastava *et al.*, *J. Virol.*, 45:555-564, 1983.

Stemmer and Klee, *Biochemistry*, 33:6859-6866, 1994.

Stockdale, *Cell Struct. Funct.*, 22:37-43, 1997.

Stratford-Perricaudet and Perricaudet, *In: Human Gene Transfer*, Eds, O. Cohen-Haguenauer and M. Boiron, Editions John Libbey Eurotext, France, pp. 51-61, 1991.

Stratford-Perricaudet *et al.*, *Hum. Gene Ther.*, 1:241-256, 1990.

Su *et al.*, *Eur. J. Biochem.*, 230:469-474, 1995.

Sullivan *et al.*,. *Circulation*, 79:324-329, 1989.

Temin, *In*: *Gene Transfer*, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188, 1986.

Tikkanen *et al.*, *Atherosclerosis*, 120:1-5, 1996.

Tikkanen *et al.*, *Atherosclerosis*, 120(1-2):1-5, 1996.

Timmerman *et al.*, *Nature*, 383:837-840, 1996.

Top *et al.*, *J. Infect. Dis.*, 124:155-160, 1971.

Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.

U.S. Patent 4,873,191

U.S. Patent 5,252,479

U.S. Patent 5,359,046

U.S. Patent 5,672,344

U.S. Patent 5,708,158

Varmus *et al.*, *Cell*, 25:23-36, 1981.

Vrbova, *J. Physiol. (Lond)*, 169:513-526, 1963.

Wagner *et al., Proc. Nat'l. Acad. Sci.*, 87(9):3410-3414, 1990.

Walsh *et al.,. Biochemistry*, 34:5561-5568, 1995.

Webster *et al., Cell*, 52:503-513, 1988.

Werthman *et al., Journal of Urology*, 155(2):753-756, 1996.

Westerblad and Allen, *J. Gen. Physiol.*, 98:615-635, 1991.

Williams and Neufer, In *The Handbook of Physiology: Integration of Motor, Circulatory, Respiratory and Metabolic Control During Exercise* (ed. L.B. Rowell and J.T.Shepard), pp.1124-1150. Bethesda:American Physiology Society, 1996.

Williams *et al., J. Comp. Physiol. [B]*, 167(8):527-535, 1997.

Williams *et al., J. Biol. Chem.*, 261:376-380 1986.

Wong *et al., Gene*, 10:87-94, 1980.

Wu & Wu, *Biochemistry*, 27:887-892, 1988.

Wu & Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.

Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.

Yamashita and Yoshioka, *J Muscle Res. Cell Motil.* 12: 37-44, 1991.

Yamazaki *et al., J. Biol. Chem.*, 271:3221-3228, 1996.

Yang *et al., Mol. Cell. Biol.*, 17:5236-5243 1997.

Yang *et al., Proc. Nat'l. Acad. Sci USA*, 87:9568-9572, 1990.

Zou *et al., J. Biol. Chem.*, 271:33592-33597, 1996.

CLAIMS:

1. A method of altering the phenotype of skeletal muscle tissue, comprising contacting said tissue with a modulator of calcineurin activity.

2. The method of claim 1, wherein the altering the phenotype comprises an increase of the proportion fast fiber to slow fiber in said tissue.

3. The method of claim 1, wherein the altering the phenotype comprises an increase of the proportion slow fiber to fast fiber in said tissue.

4. The method of claim 1, wherein said phenotype comprises a change in the size of said cells of said tissue.

5. The method of claim 1, wherein the muscle cells are derived from soleus, gastroenemius, quadriceps, tibialis anterior, pectordis, lattissimus dorsi, diaphragm, biceps, triceps, gluteus and tongue.

6. The method of claim 5, wherein the muscle cells are soleus muscle cells.

7. The method of claim 1, wherein the animal is human, murine, bovine, equine, porcine, ovine, canine, feline, rodent, avian or fish.

8. The method of claim 7, wherein the animal is a human.

9. The method of claim 1, wherein said altering the phenotype comprises an increase in expression of fiber-type specific gene expression.

10. The method of claim 9, wherein said fiber-type specific gene expression is specific to fast fiber cells.

11. The method of claim 10, wherein said gene expression comprises the expression of muscle creatinine kinase, fast myosin heavy chain, fast myosin light chain, fast troponin or parvalbumin.

12. The method of claim 9, wherein said fiber-type specific gene expression is specific to slow fiber cells.

13. The method of claim 12, wherein said gene expression comprises the expression of myoglobin, troponin I, slow myosin heavy chain, slow myosin light chain, mitochondrial proteins, GLUT4, or lipoprotein lipase.

14. The method of claim 1, wherein said modulator is an inhibitor of calcineurin activity.

15. The method of claim 13, wherein the inhibitor of calcineurin reduces the expression of calcineurin.

16. The method of claim 13, wherein the inhibitor of calcineurin is an agent that binds to and inactivates calcineurin.

17. The method of claim 13, wherein the inhibitor of calcineurin inhibits the interaction of calcineurin with an NFAT.

18. The method of claim 15, wherein the agent that reduces the expression of calcineurin is an antisense construct.

19. The method of claim 18, wherein the agent that binds to and inactivates calcineurin is an antibody or a small molecule inhibitor.

20. The method of claim 19, wherein the antibody is a single chain antibody.

21. The method of claim 19, wherein said antibody is a monoclonal antibody.

22. The method of claim 13, wherein said inhibitor is selected from the group consisting of cyclosporin, FK506, AP1510 and FK1012.

23. The method of claim 1, wherein said modulator is a stimulator of calcineurin activity.

24. The method of claim 23, wherein said stimulator of calcineurin activity increases the expression of calcineurin.

24. The method of claim 23, wherein said stimulator of calcineurin activity activates said calcineurin.

25. The method of claim 24, wherein said activator of calcineurin activity is calcium or calmodulin.

26. The method of claim 1, wherein said modulator of calcineurin activity increases the activity of an NFAT.

27. The method of claim 26, wherein said increase in an NFAT activity comprises stimulation of the dephosphorylation of an NFAT.

28. The method of claim 26, wherein said increase in an NFAT activity comprises increasing the expression of an NFAT.

29. The method of claim 26, wherein said increase in an NFAT activity comprises contacting NFAT with an agent that activates said NFAT.

30. The method of claim 26, wherein said increase in an NFAT activity comprises increasing the interaction of NFAT with MEF2.

31. The method of claim 26, wherein said NFAT is selected from the group consisting of NFAT1, NFAT2, NFAT3 and NFAT4.

32. The method of claim 1, wherein said modulator of calcineurin activity inhibits the activity of an NFAT.

33. The method of claim 32, wherein inhibition of the activity of an NFAT comprises inhibiting the dephosphorylation of NFAT.

34. The method of claim 32, wherein inhibition of the activity of an NFAT comprises reducing the expression of NFAT.

35. The method of claim 32, wherein inhibition of the activity of an NFAT comprises contacting NFAT with an agent that binds to and inactivates NFAT.

36. The method of claim 32, wherein inhibition of the activity of an NFAT comprises inhibiting the interaction of NFAT with MEF2.

37. The method of claim 34, wherein the agent that reduces the expression of an NFAT is an antisense construct.

38. The method of claim 35, wherein the agent that binds to and inactivates an NFAT is an antibody or a small molecule inhibitor.

39. The method of claim 38, wherein the antibody is a single chain antibody.

40. The method of claim 38, wherein said antibody is a monoclonal antibody.

41. The method of claim 32, wherein said NFAT is selected from the group consisting of NFAT1, NFAT2, NFAT3 and NFAT4.

42. A method of transforming a fast muscle fiber to a slow muscle fiber comprising increasing the calcineurin activity in said fast muscle fiber.

43. The method of claim 42, wherein said calcineurin is encapsulated in a liposome.

44. The method of claim 42, comprising the steps of:

(i) providing an expression construct comprising a first nucleic acid encoding an active calcineurin and promoter functional in said muscle fibers wherein said nucleic acid is under transcriptional control of said first promoter; and

(ii) contacting said expression construct with said fast muscle fiber in an amount effective to promote the activation of NFAT of said fiber;

wherein activation of NFAT in said fast fiber promotes the transformation of fast fiber to slow fiber.

45. The method of claim 42, wherein said fast fiber is located within an animal.

46. The method of claim 44, wherein said first nucleic acid is a cDNA or genomic DNA.

47. The method of claim 44, wherein said first expression construct is selected from the group consisting of an adenovirus, an adeno-associated virus, a vaccinia virus a herpes virus and lentivirus.

48. The method of claim 44, wherein said promoter is selected from the group consisting of CMV IE, SV40 IE, RSV, β-actin, tetracycline regulatable and ecdysone regulatable.

49. The method of claim 44, wherein said contacting is effected by direct injection of a muscle containing said slow fiber with said expression construct.

50. The method of claim 44, wherein said contacting comprises delivering said expression construct intravenously, subcutaneously, intramuscularly, or intraperitoneally to a muscle containing said fast fiber.

51. A method of transforming a slow muscle fiber to a fast muscle fiber comprising inhibiting calcineurin activity in said slow muscle fiber.

52. The method of claim 51, comprising the steps of:

(i) providing an expression construct comprising a first nucleic acid encoding a calcineurin gene positioned antisense to a promoter functional in said slow muscle fiber, wherein said nucleic acid is under transcriptional control of said first promoter; and

(ii) contacting said expression construct with said slow muscle fiber in an amount effective to decrease the calcineurin activity in said fiber;

wherein said decrease in calcineurin activity in said slow fiber promotes the transformation of slow fiber to fast fiber.

53. The method of claim 52, comprising contacting said slow muscle fiber with cyclosporin, FK506, AP1510 and FK1012.

54. The method of claim 52, comprising inhibiting the interaction of NFAT with MEF2.

55. A method of screening for modulators of muscle fiber phenotype comprising the steps of:

(a) providing a skeletal muscle cell expressing an NFAT and/or a MEF2 gene;

(b) contacting said cell with a candidate modulator; and

(c) monitoring said cell for a phenotype that is absent when said cell is not treated with said candidate modulator.

56. The method of claim 55, wherein said cell is in an animal.

57. The method of claim 55, wherein said cell is derived from a fast muscle cell line.

58. The method of claim 55, wherein said cell is derived from a slow muscle cell line.

59. The method of claim 55, wherein contacting is performed *in vitro*.

60. The method of claim 57, wherein said monitoring comprises measuring the activity or expression of a fast fiber-specific gene.

61. The method of claim 58, wherein said monitoring comprises measuring the activity or expression of a slow fiber-specific gene.

62. The method of claim 55, wherein said monitoring comprises measuring the size or mass of said cell.

63. The method of claim 55, wherein said monitoring comprises monitoring Ca^{++} response in said cell.

64. The method of claim 63, wherein monitoring said Ca^{++} response comprises monitoring Ca^{++} dependent gene expression in said cell.

65. The method of claim 55, wherein said contacting is performed *in vivo*.

66. The method of claim 55, wherein said candidate modulator is an antisense construct.

67. The method of claim 55, wherein said candidate modulator is from a small molecule library.

68. The method of claim 55, wherein said candidate modulator is an antibody.

69. The method of claim 68, wherein said antibody is a single chain antibody.

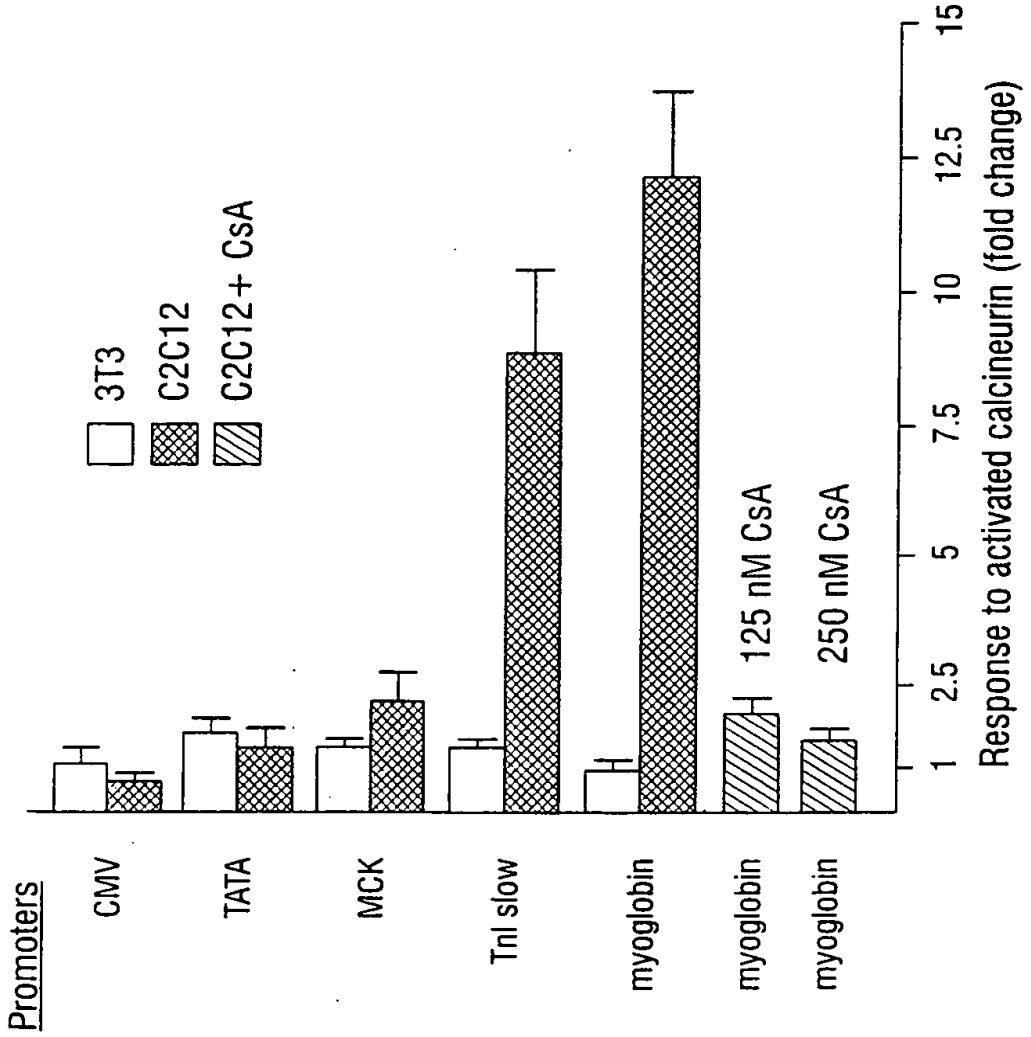


FIG. 1

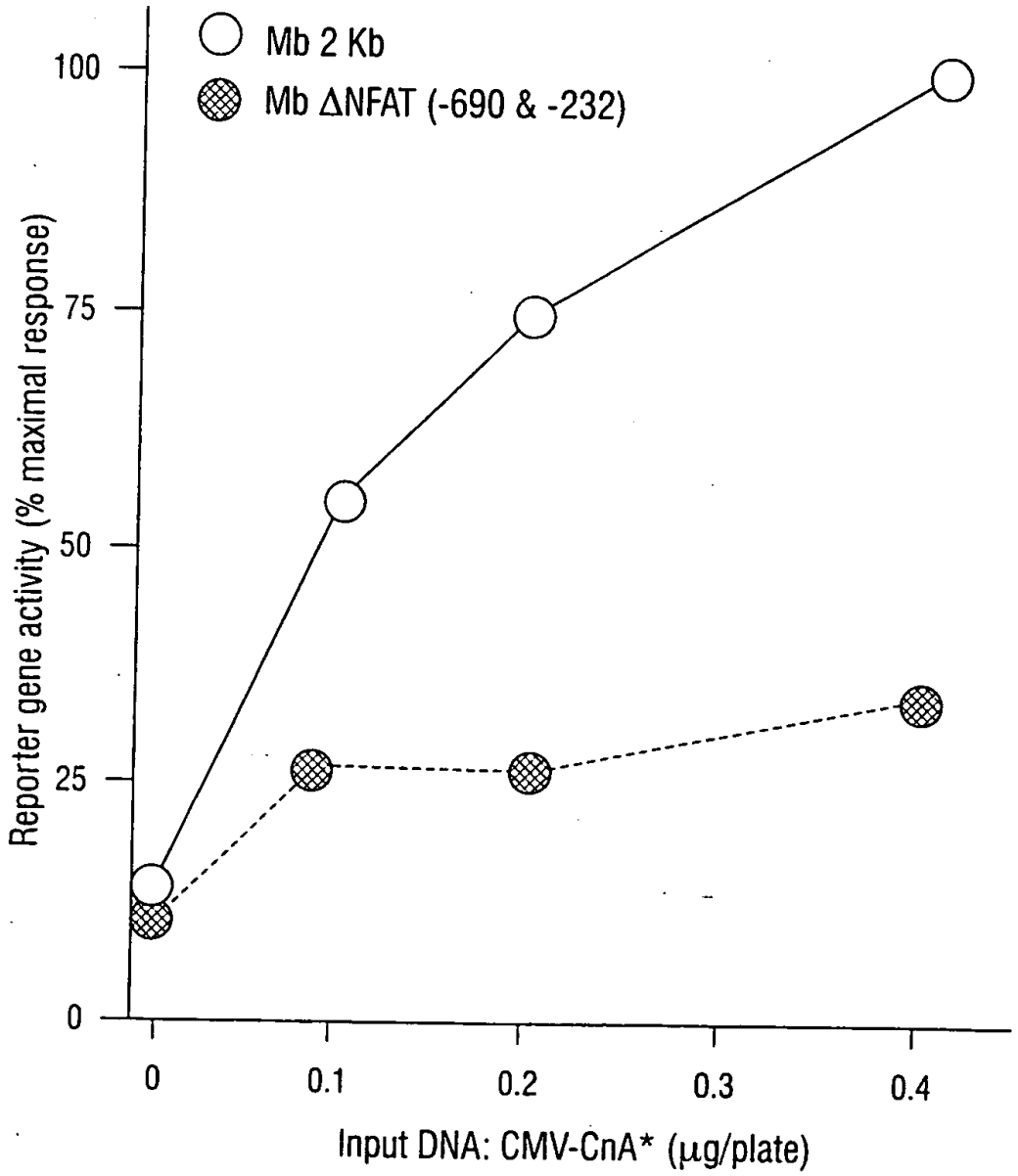


FIG. 2A

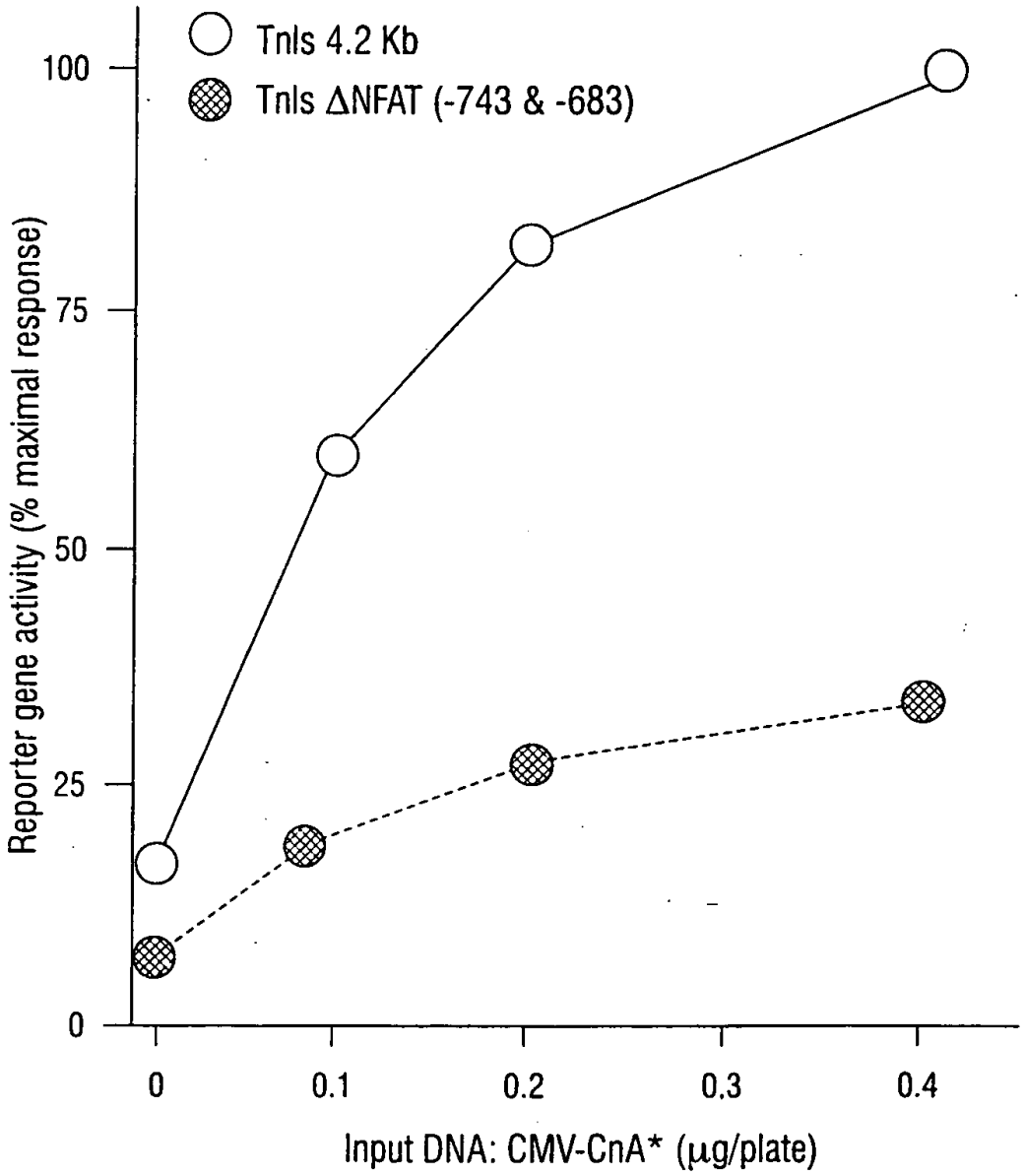


FIG. 2B

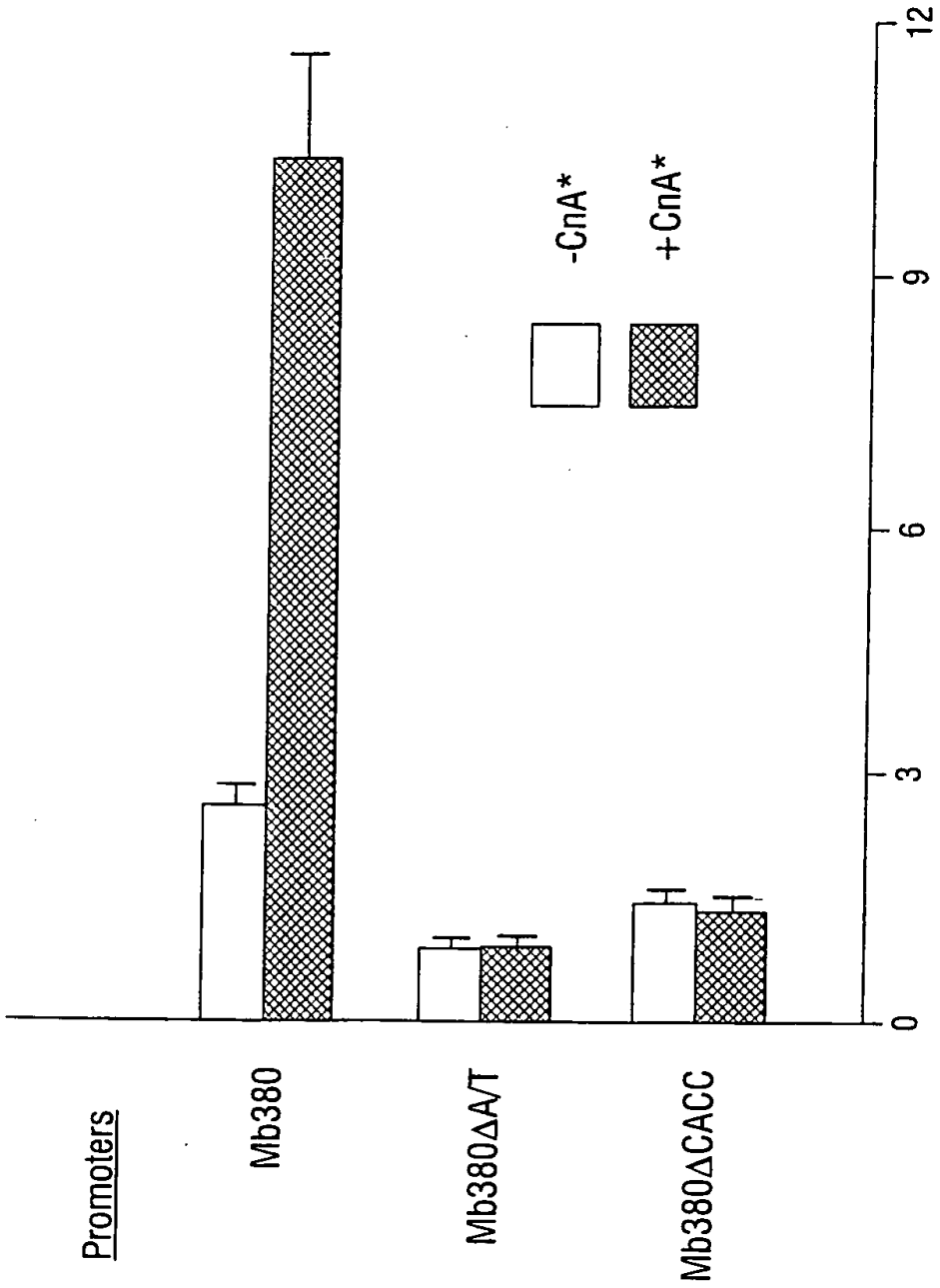


FIG. 3A

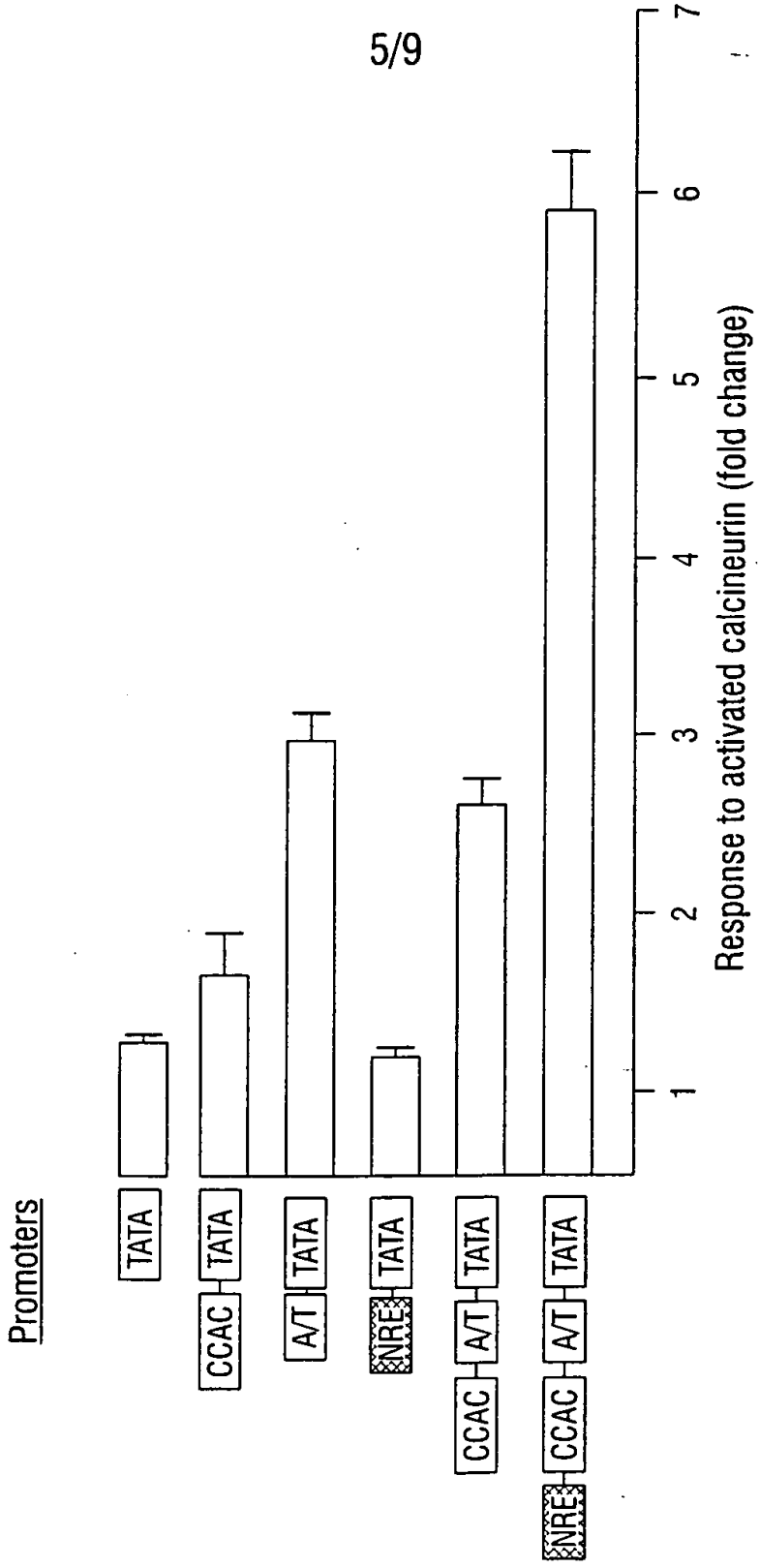


FIG. 3B

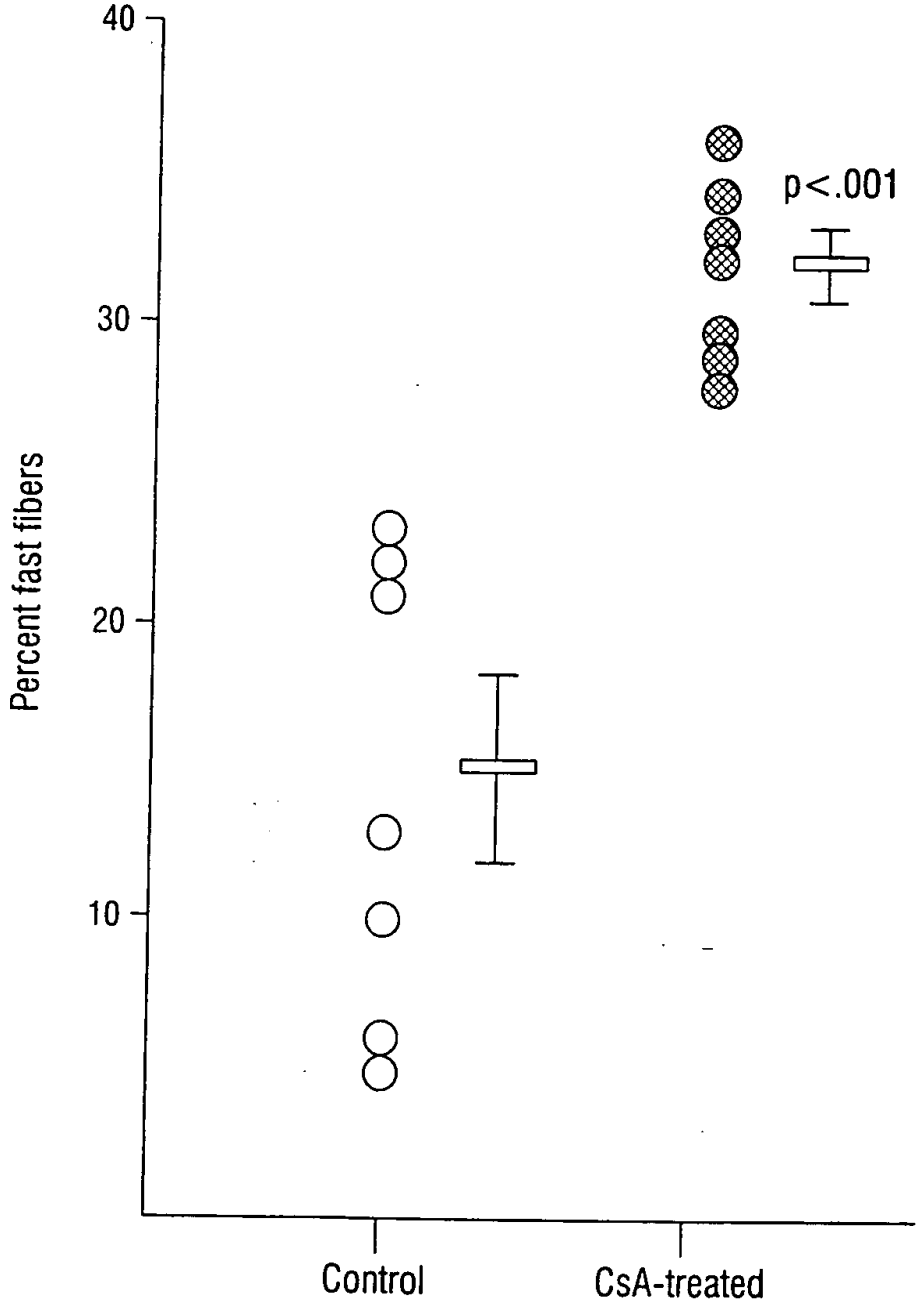


FIG. 4

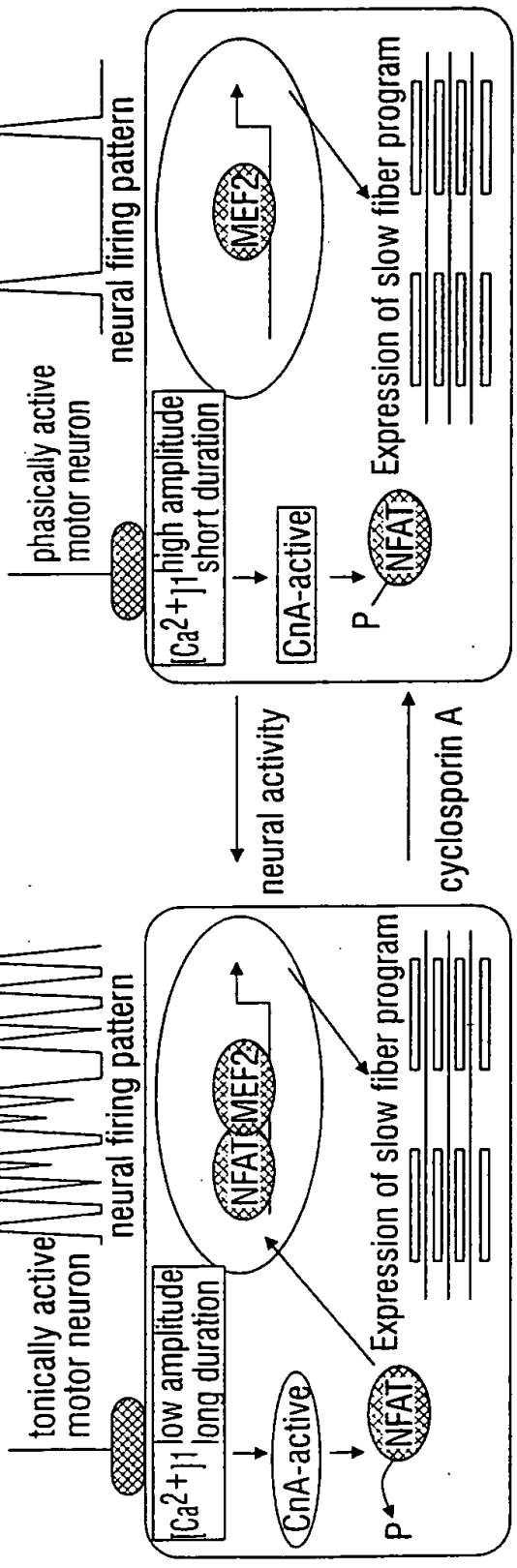


FIG. 5

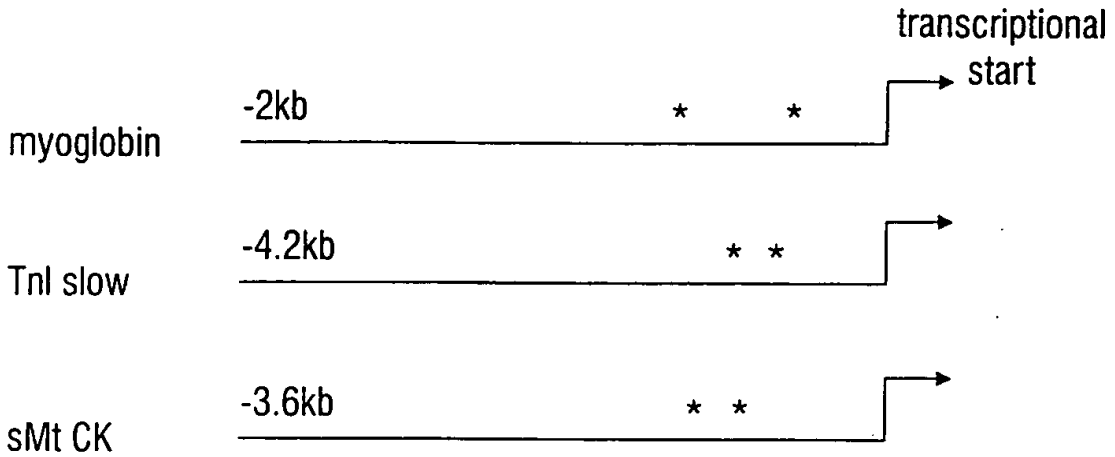


FIG. 6A-1

gene	sequence
myoglobin	-232 TGGAAAGA -690 AGGAAATA
Tnl slow	-639 TGGAAACA -738 AGGAAACC
sMt CK	-749 TGGAAACT -859 AGGAAACT
NFAT consensus:	A T GGAAANA T C

FIG. 6A-2

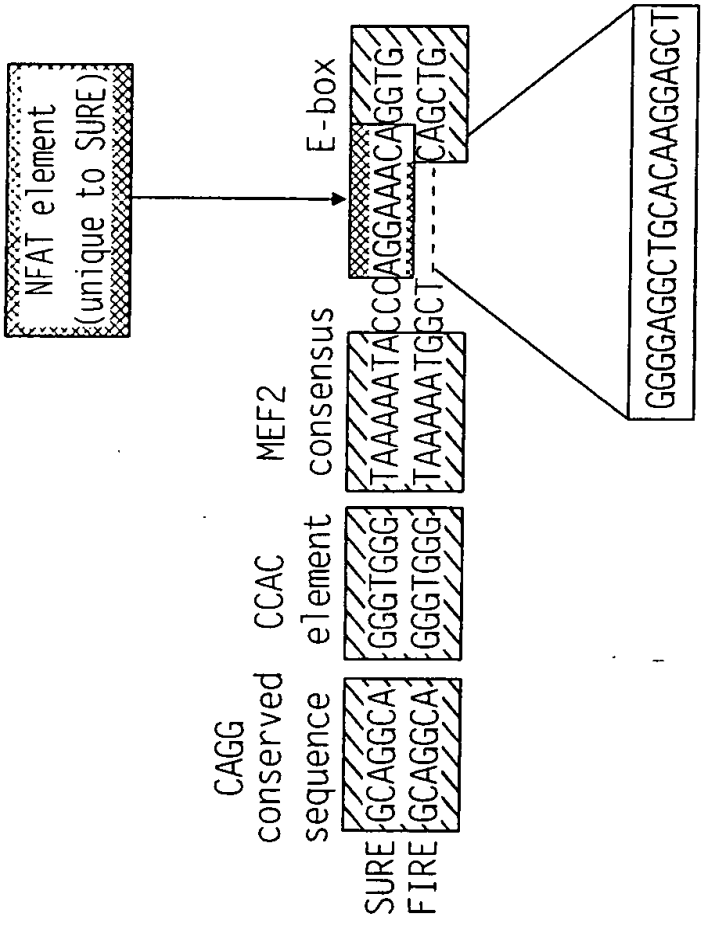


FIG. 6B

SEQUENCE LISTING

<110> Williams, R. Sanders
Olson, Eric N.

<120> CALCINEURIN-DEPENDENT CONTROL OF SKELETAL MUSCLE FIBER TYPE

<130> UTXD:562PZ1

<140> Unknown
<141> 1999-08-13

<150> 60/096,631
<151> 1998-08-14

<160> 1

<170> PatentIn Ver. 2.0

<210> 1
<211> 21
<212> DNA
<213> Homo sapiens

<400> 1
aaccaggaaa taggatgccc t 21

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 99/18439

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 33887 A (UNIV JOHNS HOPKINS MED) 6 August 1998 (1998-08-06) the whole document ---	1-69
Y	WO 95 12979 A (UNIV SOUTHERN CALIFORNIA) 18 May 1995 (1995-05-18) the whole document ---	1-69
Y	US 5 352 595 A (TAPSCOTT STEPHEN J ET AL) 4 October 1994 (1994-10-04) the whole document --- -/--	1-69

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1999	23/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hillenbrand, G

1

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/US 99/18439

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
Y	GROBET ET AL: "Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle" MAMMALIAN GENOME,US,NEW YORK, NY, vol. 9, no. 3, - March 1998 (1998-03) page 210-213 XP002085799 the whole document	1-69
Y	KAMBADUR ET AL: "Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle" GENOME RESEARCH,US,COLD SPRING HARBOR LABORATORY PRESS, vol. 7, no. 9, - September 1997 (1997-09) page 910-916 XP002085802 ISSN: 1088-9051 the whole document	1-69
P,X	WO 99 02667 A (GEORGES MICHEL ;GROBET LUC (BE); UNIV LIEGE (BE); PONCELET DOMINIQ) 21 January 1999 (1999-01-21) the whole document	1-69

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/18439

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9833887 A	06-08-1998	AU 6274298 A	25-08-1998
WO 9512979 A	18-05-1995	AU 1052795 A	29-05-1995
US 5352595 A	04-10-1994	NONE	
WO 9902667 A	21-01-1999	AU 8457198 A	08-02-1999